

## **PATENT APPLICATION**

### **Methods of Detecting Colorectal Cancer**

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## **METHODS OF DETECTING COLORECTAL CANCER**

[01] This application claims the benefit of Provisional Application 60/423,960, filed November 4, 2002, which is herein incorporated by reference in their entirety.

### **RELATED APPLICATIONS**

[02] This application is related to PCT US 01/28716, filed September 15, 2001, USSN 60/350,666 filed November 13, 2001, USSN 10/087,080 filed February 27, 2002, and USSN 60/282,698 filed April 9, 2001, USSN 60/372,246, filed April 12, 2002 each of which is herein incorporated by reference in their entirety.

### **FIELD OF THE INVENTION**

[03] The invention relates to methods of detecting antigens associated with colorectal cancer, and to the use of such antigens and their corresponding and nucleic acids for the diagnosis and prognosis evaluation of colorectal cancer. The invention further relates to methods for identifying and using candidate agents and/or targets which modulate colorectal cancer.

### **BACKGROUND OF THE INVENTION**

[04] Cancer of the colon and/or rectum (referred to as "colorectal cancer") is significant in Western populations and particularly in the United States. Cancers of the colon and rectum occur in both men and women most commonly after the age of 50, developing as the result of a pathologic transformation of normal colon epithelium to invasive cancer. Recently, a number of genetic alterations have been implicated in colorectal cancer, including mutations in tumor-suppressor genes and proto-oncogenes. Other recent work suggests that mutations in DNA repair genes also are involved in tumorigenesis. For example, inactivating mutations of both alleles of the adenomatous polyposis coli (APC) gene, a tumor suppressor gene, appears to be one of the earliest events in colorectal cancer, and may even be the initiating event. Other genes implicated in colorectal cancer include the CBF9 gene reported in U.S. Patent application 60/350,666 filed November 13, 2001, as well as the MCC gene, the p53 gene, the DCC (deleted in colorectal carcinoma) gene and other chromosome 18q genes, and

genes in the TGF- $\beta$  signaling pathway. For a review, see *Molecular Biology of Colorectal Cancer*, pp. 238-299, in *Curr. Probl. Cancer*, Sept/Oct 1997; see also Willams, *Colorectal Cancer* (1996); Kinsella & Schofield, *Colorectal Cancer: A Scientific Perspective* (1993); *Colorectal Cancer: Molecular Mechanisms, Premalignant State and its Prevention* (Schmiegel & Scholmerich eds., 2000); *Colorectal Cancer: New Aspects of Molecular Biology and Their Clinical Applications* (Hanski *et al.*, eds 2000); McArdle *et al.*, *Colorectal Cancer* (2000); Wanebo, *Colorectal Cancer* (1993); Levin, *The American Cancer Society: Colorectal Cancer* (1999); *Treatment of Hepatic Metastases of Colorectal Cancer* (Nordlinger & Jaeck eds., 1993); *Management of Colorectal Cancer* (Dunitz *et al.*, eds. 1998); *Cancer: Principles and Practice of Oncology* (Devita *et al.*, eds. 2001); *Surgical Oncology: Contemporary Principles and Practice* (Kirby *et al.*, eds. 2001); Offit, *Clinical Cancer Genetics: Risk Counseling and Management* (1997); *Radioimmunotherapy of Cancer* (Abrams & Fritzberg eds. 2000); Fleming, *AJCC Cancer Staging Handbook* (1998); *Textbook of Radiation Oncology* (Leibel & Phillips eds. 2000); and *Clinical Oncology* (Abeloff *et al.*, eds. 2000).

[05] Early diagnosis of colorectal cancer has been problematic and limited. Methods of diagnosis and prognosis testing are uncomfortable, invasive and require sample biopsy that can be time consuming. As is the case with most cancers early detection is often the key to good prognosis and cure. Therefore what is needed is a quick, convenient and effective method for detecting colorectal cancer while the cancer is still in a stage where the probability of cure is high. Accordingly, provided herein are exactly such methods as are needed for the diagnosis and prognosis determination of colorectal cancer.

## SUMMARY OF THE INVENTION

[06] The present invention provides a method of detecting colorectal cancer in a human individual. The method comprises: (a) determining the amount of one or more colorectal cancer-associated protein in a first extracellular biological sample obtained from a first human individual; and (b) comparing the amount of said one or more colorectal cancer-associated protein in said first extracellular biological sample with the amount of said one or more colorectal cancer-associated protein in an extracellular biological sample obtained from a normal human individual; whereby a higher amount of colorectal cancer-associated protein in said first extracellular biological sample indicates colorectal cancer in said first human individual. In one embodiment, the colorectal cancer-associated protein is CVA7 or CBF9.

[07] In one embodiment, a method of detecting the presence or absence of a colorectal cancer-associated protein in an extracellular biological sample, is provided. The method comprises contacting the biological sample with a binding agent which specifically binds to colorectal cancer-associated proteins selected from the group consisting of CVA7 and CBF9.

[08] In one embodiment the binding agent specifically binds CVA7. In another embodiment the binding agent specifically binds CBF9. In one embodiment, the biological sample is contacted with the binding agent that specifically binds CVA7 and the binding agent that specifically binds CBF9.

[09] In one embodiment the extracellular biological sample is selected from the group consisting of serum, whole blood, plasma, urine, saliva, sputum and cerebrospinal fluid.

[10] In one embodiment the extracellular biological sample is serum.

[11] In one embodiment, the binding agent is an antibody. In another embodiment, the antibody is a monoclonal antibody. In another embodiment the antibody is a polyclonal antibody.

[12] In one embodiment the binding agent is bound to a solid support, which may include, but is not limited to beads, dipsticks, glass, etc. In another embodiment the solid support comprises nitrocellulose. In yet another embodiment, the solid support is a well of a microtiter plate.

[13] In one embodiment, the binding agent is conjugated to a label. In one embodiment the label is radiolabel. In another embodiment the label is a fluorescent label. In another embodiment the label is a detectable enzyme. In one embodiment the detectable enzyme is alkaline phosphatase.

[14] The present invention also provides a kit for detecting the presence or absence of a colorectal cancer-associated protein in an extracellular biological sample, the kit comprising a binding agent which specifically binds to a colorectal cancer-associated protein selected from the group consisting of CVA7 and CBF9 and assay reagents for detecting the presence or absence of the colorectal cancer-associated protein in the extracellular biological sample.

[15] In one embodiment, the binding agent in the kit is labeled. In another embodiment the kit comprises the binding agent that specifically binds CVA7 and the binding agent that specifically binds CBF9.

[16] In one embodiment the binding agent supplied in the kit is an antibody. In another embodiment the antibody in the kit is a monoclonal antibody. In one embodiment the binding agent supplied in the kit is bound to a solid support.

[17] Other aspects of the invention will become apparent to the skilled artisan by the following description of the invention.

### **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows the CVA expression in colon cancer tissues and normal body atlas.

Figure 2 shows the CBF9 expression in colon cancer tissues and normal body atlas.

Figure 3 shows the detection of secreted CBF9 in control medium, Vaco-CBF9 medium, control medium plasma, Vaco-CBF9 plasma, and Vaco-CBF9 RBC.

### **DETAILED DESCRIPTION OF THE INVENTION**

#### **DEFINITIONS**

[18] The term “**extracellular biological sample**” refers to biological fluids that may be either circulating or non-circulating. Examples of circulating fluid include extracellular fluid comprising the plasma, serum, whole blood, interstitial fluid, as well as transcellular fluid such as cerebrospinal fluid, synovial fluid and pleural fluid. Examples of non-circulating fluids include, but are not limited to urine, saliva, and sputum.

[19] “**Binding agent**” refers to any substance that binds in a specific manner to another substance. For example, a binding agent may be an antibody that binds specifically to a colorectal cancer-associated CVA7 or CBF9 protein. Similarly a binding agent may be a nucleic acid that is complementary to a colorectal cancer associated CVA7 and/or CBF9 nucleic acid sequence. Alternatively, a binding agent may be a ligand specific for a particular cell surface receptor, or may also be an enzyme that binds a particular substrate. The binding agent may form an attachment that is either covalent or non-covalent, but in most cases the attachment will be non-covalent.

[20] “**Specifically binds**” means that an association between two molecular units or assemblies is selective. Specificity is judged by the magnitude of an interaction under a defined set of conditions. For example, specific binding occurs when the molecule under consideration is in direct competitive interaction with other such molecules and the other molecules cannot compete successfully with the molecule under consideration for binding of a particular substance.

[21] By “**colorectal cancer**” refers to a colon and/or rectal tumor or cancer that is classified as Dukes stage A or B as well as metastatic tumors classified as Dukes stage C or D (see, e.g., Cohen *et al.*, *Cancer of the Colon*, in *Cancer: Principles and Practice of Oncology*, pp. 1144-1197 (Devita *et al.*, eds., 5<sup>th</sup> ed. 1997); see also *Harrison's Principles of Internal*

*Medicine*, pp. 1289-129 (Wilson *et al.*, eds., 12<sup>th</sup> ed., 1991). "Treatment, monitoring, detection or modulation of colorectal cancer" includes treatment, monitoring, detection, or modulation of colorectal disease in those patients who have colorectal disease (Dukes stage A, B, C or D) in which expression of CVA7 and/or CBF9, is modulated, *e.g.* increased or decreased, indicating that the subject is more or less likely to progress to metastatic disease than a patient who does not have an increase or decrease in expression of CVA7 and/or CBF9. In Dukes stage A, the tumor has penetrated into, but not through, the bowel wall. In Dukes stage B, the tumor has penetrated through the bowel wall but there is not yet any lymph involvement. In Dukes stage C, the cancer involves regional lymph nodes. In Dukes stage D, there is distant metastasis, *e.g.*, liver, lung, etc.

[22] By the term "**recombinant nucleic acid**" herein is meant nucleic acid, originally formed in vitro, in general, by the manipulation of nucleic acid by polymerases and endonucleases, in a form not normally found in nature. Thus an isolated nucleic acid, in a linear form, or an expression vector formed in vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, *i.e.* using the in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

[23] Similarly, a "**recombinant protein**" is a protein made using recombinant techniques, *e.g.* through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of a colorectal cancer-associated protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at

increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and deletions, as discussed below.

[24] In the broadest sense, then, by “**nucleic acid**” or “**oligonucleotide**” or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage et al., *Tetrahedron* 49(10):1925 (1993) and references therein; Letsinger, *J. Org. Chem.* 35:3800 (1970); Sprinzl et al., *Eur. J. Biochem.* 81:579 (1977); Letsinger et al., *Nucl. Acids Res.* 14:3487 (1986); Sawai et al., *Chem. Lett.* 805 (1984), Letsinger et al., *J. Am. Chem. Soc.* 110:4470 (1988); and Pauwels et al., *Chemica Scripta* 26:141 (1986)), phosphorothioate (Mag et al., *Nucleic Acids Res.* 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., *J. Am. Chem. Soc.* 111:2321 (1989), O-methylphosphoroamidite linkages (see Eckstein, *Oligonucleotides and Analogues: A Practical Approach*, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, *J. Am. Chem. Soc.* 114:1895 (1992); Meier et al., *Chem. Int. Ed. Engl.* 31:1008 (1992); Nielsen, *Nature*, 365:566 (1993); Carlsson et al., *Nature* 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positively charged backbones (Denpcy et al., *Proc. Natl. Acad. Sci. USA* 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., *Angew. Chem. Intl. Ed. English* 30:423 (1991); Letsinger et al., *J. Am. Chem. Soc.* 110:4470 (1988); Letsinger et al., *Nucleoside & Nucleotide* 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, “Carbohydrate Modifications in Antisense Research”, Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., *Bioorganic & Medicinal Chem. Lett.* 4:395 (1994); Jeffs et al., *J. Biomolecular NMR* 34:17 (1994); *Tetrahedron Lett.* 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, “Carbohydrate Modifications in Antisense Research”, Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within one definition of nucleic acids (see Jenkins et al., *Chem. Soc. Rev.* (1995) pp169-176). Several nucleic acid analogs are described in Rawls, *C & E News* June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done for a

variety of reasons, for example to increase the stability and half-life of such molecules in physiological environments or as probes on a biochip.

[25] These nucleic acid analogs and mixtures of naturally occurring nucleic acids and analogs, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

[26] Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. The nucleic acids may be single stranded or double stranded, as appropriate, or contain portions of both double stranded or single stranded sequence. The depiction of a single strand ("Watson") also defines the sequence of the complementary strand ("Crick"); thus the sequences described herein also include the complement of the sequence. The nucleic acid may be DNA, genomic and cDNA, RNA or a mixed polymer, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and combinations of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides, nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as a nucleoside.

[27] By "**substantially complementary**" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions, particularly high stringency conditions, as outlined herein.

[28] "**Differential expression**," or grammatical equivalents as used herein, refers to both qualitative as well as quantitative differences in the genes' temporal and/or cellular expression patterns within and among the cells. That is, genes may be turned on or turned off in a particular state, relative to another state. A comparison of two or more states can be made. Preferably the change in expression (i.e. upregulation or downregulation) is at least about 50%, more preferably at least about 100%, more preferably at least about 150%, more preferably, at least about 200%, with from 300 to at least 1000% being especially preferred.

[29] As used herein, the terms "**colorectal cancer-associated nucleic acid**", "**colorectal cancer-associated protein**" or "**colorectal cancer-associated polynucleotide**" or "**colorectal cancer-associated transcript**" refers to nucleic acid and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have a nucleotide



sequence that has greater than about 60% nucleotide sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater nucleotide sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more nucleotides, to a CVA7 or CBF9 nucleotide sequence of Table 2; (2) bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence encoded by the CVA7 or CBF9 nucleotide sequences of Table 2, and conservatively modified variants thereof; (3) specifically hybridize under stringent hybridization conditions to a CVA7 or CBF9 nucleic acid sequence, or the complement and conservatively modified variants thereof or (4) have an amino acid sequence that has greater than about 60% amino acid sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to an amino acid sequence encoded by a CVA7 or CBF9 nucleotide sequence of Table 2. A polynucleotide or polypeptide sequence is typically from a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; cow, pig, horse, sheep, or other mammal. A “colorectal cancer-associated polypeptide” and a “colorectal cancer-associated polynucleotide,” include both naturally occurring and recombinant.

[30] Homology in this context means sequence similarity or identity, with identity being preferred. A preferred comparison for homology purposes is to compare the sequence containing sequencing errors to the correct sequence. This homology will be determined using standard techniques known in the art, including, but not limited to, the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *PNAS USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux et al., *Nucl. Acid Res.* 12:387-395 (1984), preferably using the default settings, or by inspection.

[31] In one embodiment, the sequences that are used to determine sequence identity or similarity are selected from the CVA7 or CBF9 sequences set forth in Table 2. In one embodiment the sequences utilized herein are the CVA7 and/or CBF9 sequences set forth in Table 2. In another embodiment, the sequences are naturally occurring allelic variants of the

CVA7 and/or CBF9 sequences set forth in Table 2. In another embodiment, the sequences are sequence variants as further described herein.

[32] The terms “**identical**” or percent “**identity**,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 60% identity, preferably 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection (*see, e.g.*, NCBI web site <http://www.ncbi.nlm.nih.gov/BLAST/> or the like). Such sequences are then said to be “substantially identical.” This definition also refers to, or may be applied to, the complement of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions, as well as naturally occurring, e.g., polymorphic or allelic variants, and man-made variants. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 50-100 amino acids or nucleotides in length.

[33] For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

[34] A “**comparison window**”, as used herein, includes reference to a segment of one of the number of contiguous positions selected from the group consisting typically of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA*

85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (*see, e.g., Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

[35] Preferred examples of algorithms that are suitable for determining percent sequence identity and sequence similarity include the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1997) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990). BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

[36] The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA* 90:5873-5787 (1993)).

[37] In one embodiment, the colorectal cancer-associated nucleic acids, proteins and antibodies of the invention are labeled. By “labeled” herein is meant that a compound has at least one element, isotope or chemical compound attached to enable the detection of the compound. In general, labels fall into three classes: a) isotopic labels, which may be radioactive or heavy isotopes; b) immune labels, which may be antibodies, enzymatic components, or antigens; and c) colored or fluorescent dyes. The labels may be incorporated into the colorectal cancer-associated nucleic acids, proteins and antibodies at any position. For example, the label should be capable of producing, either directly or indirectly, a detectable signal. The detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. typically the label will be conjugated to the antibody *e.g.* using a method described by Hunter *et al.*, *Nature*, 144:945 (1962); David *et al.*, *Biochemistry*, 13:1014 (1974); Pain *et al.*, *J. Immunol. Meth.*, 40:219 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407 (1982).

[38] “Antibody” refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as

gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

[39] An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain ( $V_L$ ) and variable heavy chain ( $V_H$ ) refer to these light and heavy chains respectively.

[40] Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce  $F(ab)'_2$ , a dimer of Fab which itself is a light chain joined to  $V_H$ - $C_H1$  by a disulfide bond. The  $F(ab)'_2$  may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the  $F(ab)'_2$  dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (*see Fundamental Immunology* (Paul ed., 3d ed. 1993)). While various antibody fragments are defined in terms of the digestion of an intact antibody, such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. The term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (*see, e.g., McCafferty et al., Nature* 348:552-554 (1990))

[41] A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, chemotherapy component, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

[42] A "patient" for the purposes of the present invention includes both humans and other animals, particularly mammals, and primates. The methods are applicable to both human therapy and veterinary applications. In the preferred embodiment the patient is a mammal, and in the most preferred embodiment the patient is human.

[43] The present invention provides a method for detecting colorectal cancer by determining the amount of one or more colorectal cancer-associated protein in an extracellular biological sample obtained from a human individual. The method comprises: (a) determining the amount of one or more colorectal cancer-associated protein in a first extracellular biological sample obtained from a first human individual; and (b) comparing the amount of said one or more colorectal cancer-associated protein in said first extracellular biological sample with the amount of said one or more colorectal cancer-associated protein in an extracellular biological sample obtained from a normal human individual; whereby a higher amount of colorectal cancer-associated protein in said first extracellular biological sample indicates colorectal cancer in said first human individual. In one embodiment, the colorectal cancer-associated protein is CVA7 or CBF9.

[44] A detectable amount of CVA7 and CBF9 protein in blood or serum sample from an individual indicates that the individual has colorectal cancer. The method provides a quick, convenient, and efficient method for the early detection of colorectal cancer. In addition, the methods may be used to provide a prognosis evaluation for the presence, progression, or metastasis of colorectal cancer.

[45] The present invention provides nucleic acid and protein sequences of CVA7 and CBF9. These genes are differentially expressed in colorectal cancer, and are herein termed "colorectal cancer-associated sequences". Table 2 provides the nucleic acid and protein sequences of the CVA7 and CBF9 genes as well as the Unigene and Exemplar accession numbers for CVA7 and CBF9.

[46] CBF9 has domains that suggest protein interactions. Without wishing to be bound by theory, perhaps partners may exist as blocking access to epitopes or deletional markers for cancer.

[47] In one embodiment, the colorectal cancer-associated CVA7 and CBF9 sequences are from humans; however, colorectal cancer sequences from other organisms may be useful in animal models of disease and drug evaluation or veterinary applications; thus, other colorectal cancer sequences are similarly available, from vertebrates, including mammals, including rodents (rats, mice, hamsters, guinea pigs, etc.), primates, farm animals (including sheep, goats, pigs, cows, horses, etc). Colorectal cancer sequences from other organisms may be obtained using the techniques outlined below.

[48] Colorectal cancer-associated CVA7 and CBF9 sequences can include both nucleic acid and amino acid sequences. In another embodiment, the colorectal cancer-associated

sequences are amino acid sequences. In another embodiment the colorectal cancer-associated sequences are nucleic acid sequences.

[49] A colorectal cancer-associated sequence can be initially identified by substantial nucleic acid and/or amino acid sequence homology to the CVA7 and CBF9 colorectal cancer-associated sequences provided herein. Such homology can be based upon the overall nucleic acid or amino acid sequence, and is generally determined as outlined below, using either homology programs or hybridization conditions.

[50] The nucleic acid sequences of the invention can be used to generate protein sequences, *e.g.* cloning the entire gene and verifying its frame and amino acid sequence, or by comparing it to known sequences to search for homology to provide a frame, assuming the colorectal cancer-associated protein has homology to some protein in the database being used.

[51] The present invention provides colorectal cancer-associated protein sequences. "Protein" in this sense includes proteins, polypeptides, and peptides, terms that are often used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers, those containing modified residues, and non-naturally occurring amino acid polymer.

[52] In one embodiment, the colorectal cancer-associated proteins are secreted or released proteins; the release of which can be either constitutive or regulated. These proteins may have a signal peptide or signal sequence that targets the molecule to the secretory pathway. Secreted proteins are involved in numerous physiological events; by virtue of their circulating nature, they often serve to transmit signals to various other cell types. The secreted protein may function in an autocrine manner (acting on the cell that secreted the factor), a paracrine manner (acting on cells in close proximity to the cell that secreted the factor) or an endocrine manner (acting on cells at a distance). Thus, secreted molecules find use in modulating or altering numerous aspects of physiology. Other soluble proteins may have functions related to extracellular functions, *e.g.* enzymes, or extracellular metabolic processes. Alternatively, their solubility may be indicative of a physiological abnormality. Colorectal cancer-associated proteins that are soluble proteins are particularly preferred in the present invention as they serve as good targets for diagnostic markers, for example for blood, stool, or serum tests.

[53] In one aspect, the expression levels of CVA7 and/or CBF9 genes are determined in different patient samples for which either diagnosis or prognosis information is desired, to determine whether or not a particular individual has colorectal cancer. Healthy individuals may be distinguished from individuals with colorectal cancer, and among those individuals with colorectal cancer, different prognosis states (good or poor long term survival prospects, for example) may be determined.

[54] Bioinformatics analysis of both CVA7 and CBF9 sequences predicts that these genes encode secreted proteins. Both proteins contain predicted signal sequences. CBF9 also contains von Willebrand factor (VWF) type A domains and epidermal growth factor (EGF) domains. Both of these domains are often found in secreted growth factors. Applicants have discovered that both CBF9 and CVA7 are secreted.

[55] The colorectal cancer-associated sequences of the invention can be identified as follows. Samples of serum or blood are collected from a patient. The samples are treated to extract total protein, or in some cases mRNA may be isolated. Methods for mRNA and protein isolation are known in the art. The CVA7 and CBF9 proteins can then be detected in a total protein preparation using CVA7 or CBF9 specific antibodies, or other methods known in the art. Expression data for the CVA7 and/or CBF9 proteins are thereby generated, and analysis of the data can be scrutinized to so as to provide a colorectal cancer diagnosis, or alternatively, may also be used for prognosis evaluation of an individual with colorectal cancer.

[56] Although CVA7 and/or CBF9 expression may be detected and compared between different individuals by evaluation at the gene transcript, or the protein level, evaluation at the protein level is preferred. To quantify the expression levels of CVA7 and/or CBF9, protein expression can be monitored, for example through the use of antibodies to the colorectal cancer-associated CVA7 and/or CBF9 proteins. Standard immunoassays such as ELISAs, etc., or other techniques, including mass spectroscopy assays, 2D gel electrophoresis assays, are all methods contemplated by the invention for the detection of CVA7 and/or CBF9 proteins in patient samples.

[57] In another embodiment, the CVA7 and CBF9 colorectal cancer-associated sequences are up-regulated in colorectal cancer; that is, the expression of these genes is higher in individuals with colorectal carcinoma as compared to healthy individuals. "Up-regulation" as used herein means at least about a 1.1 fold change, preferably a 1.5 or two fold change, preferably at least about a three fold change, with at least about five-fold or higher being preferred.

[58] The present invention provides novel methods for diagnosis and prognosis evaluation for colon cancer, as well as methods for screening for compositions which modulate colon cancer and compositions which bind to modulators of colon cancer. In one aspect, the expression levels of genes are determined in different patient samples for which either diagnosis or prognosis information is desired, to provide expression profiles. An expression profile of a particular sample is essentially a “fingerprint” of the state of the sample; while two states may have any particular gene similarly expressed, the evaluation of a number of genes simultaneously allows the generation of a gene expression profile that is unique to the state of the cell. That is, normal tissue may be distinguished from colon cancer tissue, and within colon cancer tissue, different prognosis states (good or poor long term survival prospects, for example) may be determined. By comparing expression profiles of colon cancer tissue in different states, information regarding which genes are important (including both up- and down-regulation of genes) in each of these states is obtained. The identification of sequences that are differentially expressed in colon cancer tissue versus normal colon tissue, as well as differential expression resulting in different prognostic outcomes, allows the use of this information in a number of ways. For example, the evaluation of a particular treatment regime may be evaluated: does a chemotherapeutic drug act to improve the long-term prognosis in a particular patient. Similarly, diagnosis may be done or confirmed by comparing patient samples with the known expression profiles. Furthermore, these gene expression profiles (or individual genes) allow screening of drug candidates with an eye to mimicking or altering a particular expression profile; for example, screening can be done for drugs that suppress the colon cancer expression profile or convert a poor prognosis profile to a better prognosis profile. This may be done by making biochips comprising sets of the important colon cancer genes, which can then be used in these screens. These methods can also be done on the protein basis; that is, protein expression levels of the colon cancer proteins can be evaluated for diagnostic and prognostic purposes or to screen candidate agents. In addition, the colon cancer nucleic acid sequences can be administered for gene therapy purposes, including the administration of antisense nucleic acids, or the colon cancer proteins (including antibodies and other modulators thereof) administered as therapeutic drugs.

[59] By comparing the expression of CVA7 and CBF9 in individuals experiencing different states of health, information regarding up- and down-regulation of CVA7 and CBF9 in each of these states is obtained. Diagnosis may then be done or confirmed. For example, does a particular patient have the CVA7 or CBF9 gene expression profile of a healthy



individual or an individual with colorectal cancer. Alternatively, one may evaluate the data to determine the likely prognosis for an individual with colorectal cancer. In some circumstances the diagnosis may involve determination of other genes in addition to CVA7 and CBF9.

### **Preparation of CVA7 and CBF9 Specific Antibodies**

#### **A. Cloning**

[60] To prepare antibodies for the serum detection of CVA7 and CBF9, mRNA is isolated from total cellular RNA by known methods. Once total RNA is isolated, mRNA is isolated by making use of the adenine nucleotide residues known as a poly (A) tail which is found on virtually every eukaryotic mRNA molecule at the 3' end thereof. Oligonucleotides composed of only deoxythymidine [oligo(dT)] are linked to cellulose and the oligo(dT)-cellulose packed into small columns. When a preparation of total cellular RNA is passed through such a column, the mRNA molecules bind to the oligo(dT) by the poly (A) tails while the rest of the RNA flows through the column. The bound mRNAs are then eluted from the column and collected.

[61] The CVA7 and CBF9 colorectal cancer-associated sequences are initially identified by substantial nucleic acid and/or amino acid sequence homology to the CVA7 and CBF9 colorectal cancer-associated sequences provided herein. Such homology can be based upon the overall nucleic acid or amino acid sequence, and is generally determined as outlined below, using either homology programs or hybridization conditions.

[62] Nucleic acid homology can be determined through hybridization studies. For example, nucleic acids that hybridize under high stringency to the nucleic acid sequences which encode the CVA7 and/or CBF9 peptides identified in Table 2, or their complements, are considered a colorectal cancer-associated sequence. High stringency conditions are known; see for example Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993).

[63] In one embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, *supra*, and Tijssen, *supra*.

[64] For selective or specific hybridization, a positive signal is typically at least two times background, preferably 10 times background hybridization. Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

[65] Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides that they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions.

[66] In addition to hybridization techniques substantial identity between two nucleic acid sequences is indicated when the polypeptide encoded by a first nucleic acid is immunologically cross-reactive with the antibodies raised against the polypeptide encoded by a second nucleic acid. Thus, a polypeptide is typically substantially identical to a second polypeptide, e.g., where the two peptides differ only by conservative substitutions.

[67] Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequences. For polymerase chain reaction (PCR), a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions are readily found in the art. In particular, protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis *et al.*, *PCR Protocols, A Guide to Methods and Applications* (1990).

#### **B. Expression of Cloned CVA7 and CBF9 Genes**

[68] In one embodiment, colorectal cancer-associated nucleic acids encoding the CVA7 and CBF9 colorectal cancer-associated proteins are used to make a variety of expression vectors to express colorectal cancer-associated proteins which can then be used in diagnostic and prognostic assays, as described below. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome.

Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the colorectal cancer-associated protein. The term “control sequences” refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, *e.g.*, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

[69] Nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

[70] The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the colorectal cancer-associated protein; *e.g.*, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the colorectal cancer-associated protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known for a variety of host cells.

[71] Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention.

[72] In addition, an expression vector may comprise additional elements. For example, an expression vector may have two replication systems, thus allowing it to be maintained in two organisms, *e.g.*, in mammalian or insect cells for expression and in a prokaryotic host for cloning and replication. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

[73] In addition, in another embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known and will vary with the host cell used.

[74] The colorectal cancer-associated proteins of the present invention are readily produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding a colorectal cancer-associated protein, under the appropriate conditions to induce or cause expression of the colorectal cancer-associated protein. The conditions appropriate for colorectal cancer-associated protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation.

[75] Appropriate host cells include yeast, bacteria, archaebacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are E. coli, Sf9 cells, C129 cells, 293 cells, BHK, CHO, COS, HeLa cells, THP1 cell line (a macrophage cell line) and human cells and cell lines.

[76] In one embodiment, the colorectal cancer-associated proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems see e.g., *"Expression of Recombinant Genes in Eukaryotic Systems"* Abelson *et al.* eds. (1999) Methods in Enzymology Vol. 306. A preferred expression vector system is a retroviral vector system such as is generally described in PCT/US97/01019 and PCT/US97/01048, both of which are hereby expressly incorporated by reference. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter. Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. Examples of transcription terminator and polyadenylation signals include those derived from SV40.

[77] Methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, are well known, and will depend upon the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

[78] In one embodiment, colorectal cancer-associated proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; *e.g.*, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. The expression vector may also include a signal peptide sequence that provides for secretion of the colorectal cancer-associated protein in bacteria. The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways. These components may be assembled into bacterial expression vectors.

[79] In one embodiment, colorectal cancer-associated proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are available.

[80] In another embodiment, colorectal cancer-associated protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillerimondii* and *P. pastoris*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*.

[81] The colorectal cancer-associated protein may also be made as a fusion protein, using available techniques. Thus, for example, for the creation of monoclonal antibodies, if the desired epitope is small, the colorectal cancer-associated protein may be fused to a carrier protein to form an immunogen. Alternatively, the colorectal cancer-associated protein may be made as a fusion protein to increase expression, or for other reasons. For example, for a colorectal cancer-associated peptide, the nucleic acid encoding the peptide may be linked to other nucleic acid for expression purposes.

[82] In addition, as is outlined herein, colorectal cancer-associated proteins can be made that are longer than the CVA7 and CBF9 depicted in Table 2 *e.g.*, by the elucidation of additional sequences, the addition of epitope or purification tags, the addition of other fusion sequences, etc.

[83] In one embodiment, the colorectal cancer-associated protein is purified or isolated after expression. Colorectal cancer-associated proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the colorectal cancer-associated protein may be purified using a standard anti-colorectal cancer antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable purification techniques, see *e.g.*, Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the colorectal cancer-associated protein. In some instances little or no purification will be necessary.

[84] Colorectal cancer-associated CVA7 and CBF9 proteins of the present invention may be shorter or longer than the wild type amino acid sequences. Thus, in one embodiment, included within the definition of colorectal cancer-associated proteins are portions or fragments of the wild type sequences. In addition, as outlined above, the colorectal cancer-associated nucleic acids of the invention may be used to obtain additional coding regions, and thus additional protein sequence, using techniques known in the art.

[85] In another embodiment, the colorectal cancer-associated proteins are derivative or variant colorectal cancer-associated proteins as compared to the wild-type sequence. That is, as outlined more fully below, the derivative colorectal cancer-associated peptide will contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the colorectal cancer-associated peptide.

[86] Also included in an embodiment of colorectal cancer-associated proteins of the present invention are amino acid sequence variants. These variants typically fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the colorectal cancer-associated protein, using cassette or PCR mutagenesis or other common techniques, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant colorectal cancer-associated protein fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring

allelic or interspecies variation of the colorectal cancer-associated protein amino acid sequence.

[87] Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

[88] Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the colorectal cancer-associated protein are desired, substitutions are generally made in accordance with the following Table 1:

**Table 1**

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

[89] Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Table 1. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

[90] The variants typically will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the colorectal cancer-associated proteins as needed. Alternatively, the variant may be designed such that the biological activity of the colorectal cancer-associated protein is altered. For example, glycosylation sites may be altered or removed.

### **C. Raising Antibodies to CVA7 and CBF9 proteins**

[91] Once expressed, and purified if necessary, the CVA7 and CBF9 colorectal cancer-associated proteins are useful in a number of applications.

[92] In one embodiment, the colorectal cancer-associated proteins of the present invention may be used to generate polyclonal and monoclonal antibodies to colorectal cancer-associated proteins, which are useful as described herein. Similarly, the colorectal cancer-associated proteins can be coupled, using standard technology, to affinity chromatography columns. These columns may then be used to purify colorectal cancer antibodies. In another embodiment, the antibodies are generated to epitopes unique to the CVA7 and CBF9 colorectal cancer-associated proteins; that is, the antibodies show little or no cross-reactivity to other proteins.

[93] In one embodiment, when the colorectal cancer-associated protein is to be used to generate antibodies, the colorectal cancer-associated protein should share at least one epitope or determinant with the full length protein. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody or T-cell receptor in the



context of MHC. Thus, in most instances, antibodies made to a smaller colorectal cancer-associated protein will be able to bind to the full length protein. In one embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity. In another embodiment, the epitope is selected from a peptide encoded by a nucleic acid of Table 2. In another preferred embodiment, the epitope is selected from the CVA7 and/or CBF9 peptide sequences.

[94] For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many techniques known in the art can be used (*see, e.g.,* Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity (*see, e.g.,* Kubly, *Immunology* (3<sup>rd</sup> ed. 1997)). Techniques for the production of single chain antibodies or recombinant antibodies (U.S. Patent 4,946,778, U.S. Patent No. 4,816,567) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express antibodies (*see, e.g.,* U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, Marks *et al.*, *Bio/Technology* 10:779-783 (1992); Lonberg *et al.*, *Nature* 368:856-859 (1994); Morrison, *Nature* 368:812-13 (1994); Fishwild *et al.*, *Nature Biotechnology* 14:845-51 (1996); Neuberger, *Nature Biotechnology* 14:826 (1996); and Lonberg & Huszar, *Intern. Rev. Immunol.* 13:65-93 (1995)). Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (*see, e.g.,* McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)). Antibodies can also be made bispecific, i.e., able to recognize two different antigens (*see, e.g.,* WO 93/08829, Traunecker *et al.*, *EMBO J.* 10:3655-3659 (1991); and Suresh *et al.*, *Methods in Enzymology* 121:210 (1986)). Antibodies can also be heteroconjugates, e.g., two covalently joined antibodies, or immunotoxins (*see, e.g.,* U.S. Patent No. 4,676,980, WO 91/00360; WO 92/200373; and EP 03089).

[95] Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the CVA7 or the CBF9 peptide of Table 2, or a peptide encoded by the CVA7 or CBF9 nucleic acids of Table 2 or fragment thereof or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thymoglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

[96] The antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature*, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized in vitro. The immunizing agent will typically include the CBF9 polypeptide or a peptide encoded by a CVA7 and/or CBF9 nucleic acid of Table 2 or a fragment thereof or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[97] The CVA7 and CBF9 colorectal cancer antibodies of the invention specifically bind to colorectal cancer-associated proteins. By “specifically bind” herein is meant that the antibodies bind to the protein with a binding constant in the range of at least  $10^{-4}$ -  $10^{-6}$   $M^{-1}$ , with a preferred range being  $10^{-7}$  -  $10^{-9}$   $M^{-1}$ . Preferred antibodies will exhibit both high affinity and high selectivity. One can screen for which exhibit low cross reactivity to other proteins *e.g.*, serum or other samples being diagnosed. For ELISA antibodies can be selected that recognize two epitopes for sandwich assay.

[98] In one embodiment the CVA7 and/or CBF9 colorectal cancer-associated proteins against which antibodies are raised are secreted proteins.

[99] Covalent modifications of colorectal cancer-associated polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a colorectal cancer-associated polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of a colorectal cancer-associated polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking colorectal cancer-associated sequences to a water-insoluble support matrix or surface for use in the method for purifying anti-colorectal cancer antibodies or screening assays, as is more fully described below. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazo-acetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidyl-propionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)-dithio]propanoate.

[100] Other modifications include deamidation of glutamyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

[101] Another type of covalent modification of the colorectal cancer-associated polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. “Altering the native glycosylation pattern” is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence colorectal cancer-associated polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence colorectal cancer-associated polypeptide.

[102] Addition of glycosylation sites to colorectal cancer-associated polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence colorectal cancer-associated polypeptide (for O-linked glycosylation sites). The colorectal cancer-associated amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the colorectal cancer-associated polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

#### **Detection of CVA7 and CBF9 in Biological Samples**

[103] In a most preferred embodiment, antibodies find use in diagnosing colorectal cancer from blood samples. As previously described, CVA7 and CBF9 colorectal cancer associated proteins may be found in circulating or non-circulating body fluids. Blood samples are convenient samples to be probed or tested for the presence of CVA7 or CBF9 colorectal cancer-associated proteins. However, other interstitial fluids, as well as cerebrospinal fluid also provide good samples in which to detect CVA7 or CBF9 proteins. Non-circulating fluids may also provide samples in which CVA7 and/or CBF9 proteins can be detected. Examples of non-circulating fluids include, but are not limited to fluids such as urine and sputum.

[104] In another embodiment CVA7 and CBF9 can be measured in biopsy samples using known histological methods.

[105] In one aspect, the expression levels of CVA7 and CBF9 gene expression are determined for different health states with respect to the colorectal cancer phenotype. Specifically, the expression levels of CVA7 and CBF9 genes in healthy individuals and in individuals with colorectal cancer are evaluated to provide understanding of the expression of CVA7 and CBF9 in colorectal cancer. There is no detectable expression of CVA7 or CBF 9 in normal colon tissues, and there is a high level expression of CVA7 or CBF9 in cancerous colon tissues. In some cases, varying severities of colorectal cancer as related to prognosis are also evaluated.

[106] It is understood that when comparing the expression of CVA7 and/or CBF9 between an individual and a standard, the skilled artisan can make a prognosis as well as a diagnosis. It is further understood that the levels of expression of CVA7 and/or CBF9 genes which indicate the diagnosis may differ from those which indicate the prognosis.

[107] In one embodiment, the colorectal cancer-associated proteins, antibodies, nucleic acids, modified proteins and cells containing colorectal cancer-associated sequences are used in prognosis assays. As above, expression of CVA7 and CBF9 may be correlated to colorectal cancer severity, in terms of long-term prognosis. Again, this may be done on either a protein or gene level, with the use of proteins being preferred.

[108] Antibodies can be used to detect the colorectal cancer-associated CVA7 and CBF9 proteins by any of the previously described immunoassay techniques including ELISA, immunoblotting (Western blotting), immunoprecipitation, BIACORE technology and the like, as will be appreciated by one of ordinary skill in the art.

[109] In another embodiment, binding assays are done. In general, purified or isolated gene product is used; that is, the gene products of CVA7 and/or CBF9 nucleic acids are made. In general, this is done as is known in the art. For example, antibodies are generated to the protein gene products, and standard immunoassays are run to determine the amount of protein present.

[110] Positive controls and negative controls may be used in the assays. Preferably all control and test samples are performed in at least triplicate to obtain statistically significant results. Incubation of all samples is for a time sufficient for the binding of the agent to the protein. Following incubation, all samples are washed free of non-specifically bound material and the amount of bound, generally labeled agent determined. For example, where a radiolabel is employed, the samples may be counted in a scintillation counter to determine the amount of bound compound.

[111] Once the assay is run, the data is analyzed to determine the expression levels, and changes in expression levels between healthy individuals and those individuals with colorectal cancer, or between individuals with different severities of colorectal cancer disease are compared.

[112] As will be appreciated by those in the art, nucleic acid and protein binding agents can be attached or immobilized to a solid support. This can be accomplished in a wide variety of ways. By "immobilized" and grammatical equivalents herein is meant the association or binding between the nucleic acid probe, antibody, or other binding agent and the solid support is sufficient to be stable under the conditions of binding, washing, analysis, and removal as outlined below. The binding between the binding agent and the support can be covalent or non-covalent. By "non-covalent binding" and grammatical equivalents herein is meant one or more of electrostatic, hydrophilic, and hydrophobic interactions. Included in non-covalent binding is the covalent attachment of a molecule, such as, streptavidin to the

support and the non-covalent binding of the biotinylated binding agent to the streptavidin. By "covalent binding" and grammatical equivalents herein is meant that the two moieties, the solid support and the binding agent, are attached by at least one bond, including sigma bonds, pi bonds and coordination bonds. Covalent bonds can be formed directly between the binding agent and the solid support or can be formed by a cross linker or by inclusion of a specific reactive group on either the solid support or the binding agent or both molecules. Immobilization may also involve a combination of covalent and non-covalent interactions.

[113] In one embodiment, the oligonucleotides are synthesized as is known in the art, and then attached to the surface of the solid support. As will be appreciated by those skilled in the art, either the 5' or 3' terminus may be attached to the solid support, or attachment may be via an internal nucleoside. A nucleic acid probe that is functional as a binding agent in the present invention is generally single stranded but can be partially single and partially double stranded. The strandedness of the probe is dictated by the structure, composition, and properties of the target sequence. In general, the nucleic acid probes range from about 8 to about 100 bases long, with from about 10 to about 80 bases being preferred, and from about 30 to about 50 bases being particularly preferred. That is, generally whole genes are not used. In some embodiments, much longer nucleic acids can be used, up to hundreds of bases.

[114] In one embodiment, the binding agent immobilized to a solid support is an antibody. In this case antibodies may be derivatized with bifunctional agents for the purpose of crosslinking antibodies to CVA7 and CBF9 colorectal cancer-associated sequences to a water-insoluble support matrix or surface for use in the method for identifying CVA7 and/or CBF9 proteins in serum or blood samples. Commonly used crosslinking agents include, e.g., 1,1-bis(diazo-acetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidyl-propionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)-dithio]propanoate.

### **Kits for Use in Diagnostic and/or Prognostic Applications**

[115] For use in diagnostic, research, and therapeutic applications suggested above, kits are also provided by the invention. In the diagnostic and research applications such kits may include any or all of the following: assay reagents, buffers, colorectal cancer-specific nucleic acids or antibodies, hybridization probes and/or primers, antisense polynucleotides,

ribozymes, dominant negative ovarian cancer polypeptides or polynucleotides, small molecules inhibitors of colorectal cancer-associated sequences *etc.* A therapeutic product may include sterile saline or another pharmaceutically acceptable emulsion and suspension base.

[116] In addition, the kits may include instructional materials containing directions (i.e., protocols) for the practice of the methods of this invention. While the instructional materials typically comprise written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

[117] The present invention also provides for kits for screening for modulators of colorectal cancer-associated sequences. Such kits can be prepared from readily available materials and reagents. For example, such kits can comprise one or more of the following materials: a colorectal cancer-associated polypeptide or polynucleotide, reaction tubes, and instructions for testing colorectal cancer-associated activity. Optionally, the kit contains biologically active colorectal cancer protein. A wide variety of kits and components can be prepared according to the present invention, depending upon the intended user of the kit and the particular needs of the user. Diagnosis would typically involve evaluation of a plurality of genes or products. The genes will be selected based on correlations with important parameters in disease.

## EXAMPLES

### **Example 1: Tissue Preparation, Labeling Chips, and Fingerprints**

#### **Purifying total RNA from tissue sample using TRIzol Reagent**

[118] The tissue sample weight is first estimated. The tissue samples are homogenized in 1 ml of TRIzol per 50 mg of tissue using a homogenizer (e.g., Polytron 3100). The size of the generator/probe used depends upon the sample amount. A generator that is too large for the amount of tissue to be homogenized will cause a loss of sample and lower RNA yield. A larger generator (e.g., 20 mm) is suitable for tissue samples weighing more than 0.6 g. Fill tubes should not be overfilled. If the working volume is greater than 2 ml and no greater than 10 ml, a 15 ml polypropylene tube (Falcon 2059) is suitable for homogenization.

[119] Tissues should be kept frozen until homogenized. The TRIzol is added directly to the frozen tissue before homogenization. Following homogenization, the insoluble material is removed from the homogenate by centrifugation at 7500 x g for 15 min. in a Sorvall superspeed or 12,000 x g for 10 min. in an Eppendorf centrifuge at 4°C. The cleared homogenate is then transferred to a new tube(s). Samples may be frozen and stored at -60 to -70°C for at least one month or else continue with the purification.

[120] The next process is phase separation. The homogenized samples are incubated for 5 minutes at room temperature. Then, 0.2 ml of chloroform per 1 ml of TRIzol reagent is added to the homogenization mixture. The tubes are securely capped and shaken vigorously by hand (do not vortex) for 15 seconds. The samples are then incubated at room temp. for 2-3 minutes and next centrifuged at 6500 rpm in a Sorvall superspeed for 30 min. at 4°C.

[121] The next process is RNA Precipitation. The aqueous phase is transferred to a fresh tube. The organic phase can be saved if isolation of DNA or protein is desired. Then 0.5 ml of isopropyl alcohol is added per 1 ml of TRIzol reagent used in the original homogenization. Then, the tubes are securely capped and inverted to mix. The samples are then incubated at room temp. for 10 minutes and centrifuged at 6500 rpm in Sorvall for 20 min. at 4°C.

[122] The RNA is then washed. The supernatant is poured off and the pellet washed with cold 75% ethanol. 1 ml of 75% ethanol is used per 1 ml of the TRIzol reagent used in the initial homogenization. The tubes are capped securely and inverted several times to loosen pellet without vortexing. They are next centrifuged at <8000 rpm (<7500 x g) for 5 minutes at 4°C.

[123] The RNA wash is decanted. The pellet is carefully transferred to an Eppendorf tube (sliding down the tube into the new tube by use of a pipet tip to help guide it in if necessary). Tube(s) sizes for precipitating the RNA depending on the working volumes. Larger tubes



may take too long to dry. Dry pellet. The RNA is then resuspended in an appropriate volume (e.g., 2 -5 ug/ul) of DEPC H<sub>2</sub>O. The absorbance is then measured.

[124] The poly A+ mRNA may next be purified from total RNA by other methods such as Qiagen's RNEASY® (chromatographic materials for separation of nucleic acids) kit. The poly A + mRNA is purified from total RNA by adding the OLIGOTEX® (chemicals for the purification of nucleic acids) suspension which has been heated to 37oC and mixing prior to adding to RNA. The Elution Buffer is incubated at 70oC. If there is precipitate in the buffer, warm up the 2 x Binding Buffer at 65oC. The total RNA is mixed with DEPC-treated water, 2 x Binding Buffer, and OLIGOTEX® (chemicals for the purification of nucleic acids) according to Table 2 on page 16 of the OLIGOTEX® Handbook and next incubated for 3 minutes at 65oC and 10 minutes at room temperature.

[125] The preparation is centrifuged for 2 minutes at 14,000 to 18,000 xg, preferably, at a "soft setting." The supernatant is removed without disturbing Oligotex pellet. A little bit of solution can be left behind to reduce the loss of OLIGOTEX®. The supernatant is saved until satisfactory binding and elution of poly A+ mRNA has been found.

[126] Then, the preparation is gently resuspended in Wash Buffer OW2 and pipetted onto the spin column and centrifuged at full speed (soft setting if possible) for 1 minute.

[127] Next, the spin column is transferred to a new collection tube and gently resuspended in Wash Buffer OW2 and centrifuged as described herein.

[128] Then, the spin column is transferred to a new tube and eluted with 20 to 100 ul of preheated (70oC) Elution Buffer. The OLIGOTEX® resin is gently resuspended by pipetting up and down. The centrifugation is repeated as above and the elution repeated with fresh elution buffer or first eluate to keep the elution volume low.

[129] The absorbance is next read to determine the yield, using diluted Elution Buffer as the blank.

[130] Before proceeding with cDNA synthesis, the mRNA is precipitated before proceeding with cDNA synthesis, as components leftover or in the Elution Buffer from the OLIGOTEX® purification procedure will inhibit downstream enzymatic reactions of the mRNA. 0.4 vol. of 7.5 M NH<sub>4</sub>OAc + 2.5 vol. of cold 100% ethanol is added and the preparation precipitated at -20oC 1 hour to overnight (or 20-30 min. at -70oC), and centrifuged at 14,000-16,000 x g for 30 minutes at 4oC. Next, the pellet is washed with 0.5 ml of 80% ethanol (-20oC) and then centrifuged at 14,000-16,000 x g for 5 minutes at room temperature. The 80% ethanol wash is then repeated. The last bit of ethanol from the pellet

is then dried without use of a speed vacuum and the pellet is then resuspended in DEPC H<sub>2</sub>O at 1 µg/µl concentration.

**Alternatively the RNA may be purified using other methods (e.g., Qiagen's RNEASY<sup>®</sup> kit).**

[131] No more than 100 µg is added to the RNEASY<sup>®</sup> (chromatographic materials for separation of nucleic acids) column. The sample volume is adjusted to 100 µl with RNase-free water. 350 µl Buffer RLT and then 250 µl ethanol (100%) are added to the sample. The preparation is then mixed by pipetting and applied to an RNEASY<sup>®</sup> mini spin column for centrifugation (15 sec at >10,000 rpm). If yield is low, reapply the flowthrough to the column and centrifuge again.

[132] Then, transfer column to a new 2 ml collection tube and add 500 µl Buffer RPE and centrifuge for 15 sec at >10,000 rpm. The flowthrough is discarded. 500 µl Buffer RPE and is then added and the preparation is centrifuged for 15 sec at >10,000 rpm. The flowthrough is discarded, and the column membrane dried by centrifuging for 2 min at maximum speed. The column is transferred to a new 1.5-ml collection tube. 30-50 µl of RNase-free water is applied directly onto column membrane. The column is then centrifuged for 1 min at >10,000 rpm and the elution step repeated.

[133] The absorbance is then read to determine yield. If necessary, the material may be ethanol precipitated with ammonium acetate and 2.5X volume 100% ethanol.

#### **First Strand cDNA Synthesis**

[134] The first strand can be made using Gibco's "SUPERScript<sup>®</sup> Choice System for cDNA Synthesis" kit. The starting material is 5 µg of total RNA or 1 µg of polyA+ mRNA. For total RNA, 2 µl of SUPERScript<sup>®</sup> RT is used; for polyA+ mRNA, 1 µl of SUPERScript<sup>®</sup> RT is used. The final volume of first strand synthesis mix is 20 µl. The RNA should be in a volume no greater than 10 µl. The RNA is incubated with 1 µl of 100 pmol T7-T24 oligo for 10 min at 70°C followed by addition on ice of 7 µl of: 4 µl 5X 1<sup>st</sup> Strand Buffer, 2 µl of 0.1M DTT, and 1 µl of 10mM dNTP mix. The preparation is then incubated at 37°C for 2 min before addition of the SUPERScript<sup>®</sup> RT followed by incubation at 37°C for 1 hour.

### **Second Strand Synthesis**

[135] For the second strand synthesis, place 1st strand reactions on ice and add: 91 ul DEPC H<sub>2</sub>O; 30 ul 5X 2nd Strand Buffer; 3 ul 10mM dNTP mix; 1 ul 10 U/ul E.coli DNA Ligase; 4 ul 10 U/ul E.coli DNA Polymerase; and 1 ul 2 U/ul RNase H. Mix and incubate 2 hours at 16°C. Add 2 ul T4 DNA Polymerase. Incubate 5 min at 16°C. Add 10 ul of 0.5M EDTA.

### **Cleaning up cDNA**

[136] The cDNA is purified using Phenol:Chloroform:Isoamyl Alcohol (25:24:1) and Phase-Lock gel tubes. The PLG tubes are centrifuged for 30 sec at maximum speed. The cDNA mix is then transferred to PLG tube. An equal volume of phenol:chloroform:isamyl alcohol is then added, the preparation shaken vigorously (no vortexing), and centrifuged for 5 minutes at maximum speed. The top aqueous solution is transferred to a new tube and ethanol precipitated by adding 7.5X 5M NH<sub>4</sub>OAc and 2.5X volume of 100% ethanol. Next, it is centrifuged immediately at room temperature for 20 min, maximum speed. The supernatant is removed, and the pellet washed with 2X with cold 80% ethanol. As much ethanol wash as possible should be removed before air drying the pellet; and resuspending it in 3 ul RNase-free water.

### **In vitro Transcription (IVT) and labeling with biotin**

[137] In vitro Transcription (IVT) and labeling with biotin is performed as follows: Pipet 1.5 ul of cDNA into a thin-wall PCR tube. Make NTP labeling mix by combining 2 ul T7 10xATP (75 mM) (Ambion); 2 ul T7 10xGTP (75 mM) (Ambion); 1.5 ul T7 10xCTP (75 mM) (Ambion); 1.5 ul T7 10xUTP (75 mM) (Ambion); 3.75 ul 10 mM Bio-11-UTP (Boehringer-Mannheim/Roche or Enzo); 3.75 ul 10 mM Bio-16-CTP (Enzo); 2 ul 10x T7 transcription buffer (Ambion); and 2 ul 10x T7 enzyme mix (Ambion). The final volume is 20 ul. Incubate 6 hours at 37°C in a PCR machine. The RNA can be furthered cleaned. Clean-up follows the previous instructions for RNEASY<sup>®</sup> columns or Qiagen's RNeasy protocol handbook. The cRNA often needs to be ethanol precipitated by resuspension in a volume compatible with the fragmentation step.

[138] Fragmentation is performed as follows. 15 ug of labeled RNA is usually fragmented. Try to minimize the fragmentation reaction volume; a 10 ul volume is recommended but 20 ul is all right. Do not go higher than 20 ul because the magnesium in the fragmentation buffer contributes to precipitation in the hybridization buffer. Fragment RNA by incubation at 94 C for 35 minutes in 1 x Fragmentation buffer (5 x Fragmentation buffer is 200 mM

Tris-acetate, pH 8.1; 500 mM KOAc; 150 mM MgOAc). The labeled RNA transcript can be analyzed before and after fragmentation. Samples can be heated to 65°C for 15 minutes and electrophoresed on 1% agarose/TBE gels to get an approximate idea of the transcript size range.

[139] For hybridization, 200  $\mu$ l (10  $\mu$ g cRNA) of a hybridization mix is put on the chip. If multiple hybridizations are to be done (such as cycling through a 5 chip set), then it is recommended that an initial hybridization mix of 300  $\mu$ l or more be made. The hybridization mix is: fragment labeled RNA (50 ng/ $\mu$ l final conc.); 50 pM 948-b control oligo; 1.5 pM BioB; 5 pM BioC; 25 pM BioD; 100 pM CRE; 0.1 mg/ml herring sperm DNA; 0.5 mg/ml acetylated BSA; and 300  $\mu$ l with 1xMES hyb buffer.

[140] The hybridization reaction is conducted with non-biotinylated IVT (purified by RNEASY<sup>®</sup> columns) (see example 1 for steps from tissue to IVT): The following mixture is prepared:

IVT antisense RNA; 4 $\mu$ g:	$\mu$ l
Random Hexamers (1 $\mu$ g/ $\mu$ l):	4 $\mu$ l
H <sub>2</sub> O:	<u>          <math>\mu</math>l          </u>
	14 $\mu$ l

Incubate the above 14  $\mu$ l mixture at 70°C for 10 min.; then put on ice.

The Reverse transcription procedure uses the following mixture:

0.1 M DTT:	3 $\mu$ l
50X dNTP mix:	0.6 $\mu$ l
H <sub>2</sub> O:	2.4 $\mu$ l
Cy3 or Cy5 dUTP (1mM):	3 $\mu$ l
SS RT II (BRL):	1 $\mu$ l
	<u>          <math>\mu</math>l          </u>
	16 $\mu$ l

The above solution is added to the hybridization reaction and incubated for 30 min., 42°C.

Then, 1  $\mu$ l SSII is added and incubated for another hour before being placed on ice.

[141] The 50X dNTP mix contains 25mM of cold dATP, dCTP, and dGTP, 10mM of dTTP and is made by adding 25  $\mu$ l each of 100mM dATP, dCTP, and dGTP; 10  $\mu$ l of 100mM dTTP to 15  $\mu$ l H<sub>2</sub>O.

[142] RNA degradation is performed as follows. Add 86 µl H<sub>2</sub>O, 1.5 µl 1M NaOH/ 2 mM EDTA and incubate at 65°C, 10 min.. For U-Con 30, 500 µl TE/sample spin at 7000 g for 10 min, save flow through for purification. For Qiagen purification, suspend u-con recovered material in 500 µl buffer PB and proceed using Qiagen protocol. For DNase digestion, add 1 µl of 1/100 dilution of DNase/30 µl Rx and incubate at 37°C for 15 min. Incubate at 5 min 95°C to denature the DNase.

### **Sample preparation**

[143] For sample preparation, add Cot-1 DNA, 10 µl; 50X dNTPs, 1 µl; 20X SSC, 2.3 µl; Na pyro phosphate, 7.5 µl; 10 mg/ml Herring sperm DNA; 1 µl of 1/10 dilution to 21.8 final vol. Dry in speed vac. Resuspend in 15 µl H<sub>2</sub>O. Add 0.38 µl 10% SDS. Heat 95°C, 2 min and slow cool at room temp. for 20 min. Put on slide and hybridize overnight at 64°C. Washing after the hybridization: 3X SSC/0.03% SDS: 2 min., 37.5 ml 20X SSC+0.75ml 10% SDS in 250ml H<sub>2</sub>O; 1X SSC: 5 min., 12.5 ml 20X SSC in 250ml H<sub>2</sub>O; 0.2X SSC: 5 min., 2.5 ml 20X SSC in 250ml H<sub>2</sub>O. Dry slides and scan at appropriate PMT's and channels.

### **Example 2: Expression data on colon cancers and normal tissues.**

[144] Expression studies of colon tissues and other normal tissues were performed according to Example 1. Figure 1 shows the CVA expression in colon cancer tissues and normal body atlas. Figure 2 shows the CBF9 expression in colon cancer tissues and normal body atlas.

### **Example 3. Detection of Secreted CBF9 and CVA7**

[145] His-tagged versions of the genes for CBF9 and CVA7 were transfected into a colon cancer cell line (Vaco 364). These cell lines were then grown in tissue culture *in vitro* and as xenografts in severe combined immunodeficient (SCID) mice *in vivo*. The media from the cells grown *in vitro* and mouse serum from animals bearing xenograft tumors were then analyzed for the presence of secreted protein. To detect secreted protein, an antibody that binds to the His-tag on the recombinant proteins was used. Our results show that both CVA7 and CBF9 were secreted into the media by transfected cells grown in culture, but not in control cells that did not express the target genes. Similarly, both proteins were detected in the serum of mice carrying tumors of transfected cells, but not in the serum of control mice.

Figure 3 shows the detection of secreted CBF9 in Vaco-CBF9 medium, Vaco-CBF9 plasma, and Vaco-CBF9 RBC, but not in control medium, or control medium plasma.

**Example 3: Analysis of CVA7 and CBF9 Expression in Blood Using Antibody-sandwich ELISA to Detect the Soluble Antigens**

[146] Blood samples are obtained from a patient using methods outlined in U.S. Patent 6,283, 926, the content of which is herein incorporated by reference.

[147] Molecular profiles of various serum and blood samples are determined by performance of antibody-sandwich ELISA to detect the soluble antigens. Methods for conducting antibody-sandwich ELISA can be found in: *Current Protocols in Molecular Biology* (1998) Vol. 2, page 11.2.8 F.M. Ausubel *et al.* eds.

[148] Detection of CVA7 and/or CBF9 protien are diagnostic of colorectal cancer.

[149] It is understood that the examples described above in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All publications, sequences of accession numbers, and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

**TABLE 2: CBF9 and CVA7 DNA and Protein Sequences**

Table 2 shows the nucleotide and protein sequences for CBF9 and CVA7 genes. The CVA7 sequences shown here comprise two sequence variants of the gene.

**CBF9 DNA sequence (SEQ ID NO: 1)**

Unigene number: Hs.157601  
 Probeset Accession #: W07459  
 Nucleic Acid Accession #: AC005383  
 Coding Sequence: 328-2751 (underlined sequences correspond to start and stop codons)

1	11	21	31	41	51	
GACAGTGTTC	GCGGCTGCAC	CGCTCGGAGG	CTGGGTGACC	CGCGTAGAAG	TGAAGTACTT	60
TTTTATTTGC	AGACCTGGGC	CGATGCCGCT	TTAAAAACG	CGAGGGGCTC	TATGCACCTC	120
CCTGGCGGTA	GTTCCCTCCGA	CCTCAGCCGG	GTCGGGTCGT	GCCGCCCTCT	CCCAGGAGAG	180
ACAAACAGGT	GTCCCACGTG	GCAGCCGCGC	CCCCGGCGCC	CCTCCTGTGA	TCCCGTAGCG	240
CCCCCTGGCC	CGAGCCGCGC	CCGGGTCTGT	GAGTAGAGCC	CCCCGGGCAC	CGAGCGCTGG	300
TCGCCGCTCT	CCTTCCGTTA	TATCAACATG	CCCCCTTTCC	TGTGCTGGA	GGCCGCTCTG	360
GTTTTCTGT	TTTCCAGAGT	GCCCCATCT	CTCCCTCTCC	AGGAAGTCCA	TGTAAGCAAA	420
GAAACCATCG	GGAAGATTTC	AGCTGCCAGC	AAAATGATGT	GGTGCTCGGC	TGCACTGGAC	480
ATCATGTTTC	TGTTAGATGG	GTCTAACAGC	GTCGGGAAAG	GGAGCTTTGA	AAGGTCCAAG	540
CACTTTGCCA	TCACAGTCTG	TGACGGTCTG	GACATCAGCC	CCGAGAGGGT	CAGAGTGGGA	600
GCATTCCAGT	TCAGTTCCAC	TCCTCATCTG	GAATTCCCCT	TGGATTCATT	TTCAACCCAA	660
CAGGAAGTGA	AGGCAAGAAAT	CAAGAGGATG	GTTTTCAAAG	GAGGGCGCAC	GGAGACGGAA	720
CTTGCTCTGA	AATACCTTCT	GCACAGAGGG	TTCCTGGAG	GCAGAAATGC	TTCTGTGCCC	780
CAGATCCTCA	TCATCGTTCAC	TGATGGGAAG	CCCCAGGGGG	ATGTGGCACT	GCCATCCAAG	840
CAGCTGAAGG	AAAGGGGTGT	CACTGTGTTT	GCTGTGGGGG	TCAGGTTTCC	CAGGTGGGAG	900
GAGCTGCATG	CACTGGCCAG	CGAGCCTAGA	GGCAGCACG	TGCTGTTGGC	TGAGCAGGTG	960
GAGGATGCCA	CCAACGGCCT	CTTCAGCACC	CTCAGCAGCT	CGGCCATCTG	CTCCAGCGCC	1020
ACGCCAGACT	GCAGGGTCGA	GGCTCACCCC	TGTGAGCACA	GGACGCTGGA	GATGGTCCGG	1080
GAGTTCGCTG	GCAATGCCCC	ATGCTGGAGA	GGATCGCGGC	GGACCCCTGC	GGTGCTGGCT	1140
GCACACTGTC	CCTTCTACAG	CTGGAAGAGA	GTGTTCTTAA	CCCACCCTGC	CACCTGCTAC	1200
AGGACCACCT	GCCCAGGCC	CTGTGACTCG	CAGCCCTGCC	AGAATGGAGG	CACATGTGTT	1260
CCAGAAGGAC	TGGACGGCTA	CCAGTGCCTC	TGCCCGCTGG	CCTTTGGAGG	GGAGGCTAAC	1320
TGTGCCCTGA	AGCTGAGCCT	GGAATGCAGG	GTCGACCTCC	TCTTCCTGCT	GGACAGCTCT	1380
GCGGGCACCA	CTCTGGACGG	CTTCCTGCGG	GCCAAAGTCT	TCGTGAAGCG	GTTTGTGCGG	1440
GCCGTGCTGA	GCGAGGACTC	TCGGGCCCGA	GTGGGTGTGG	CCACATACAG	CAGGGAGCTG	1500
CTGGTGGCGG	TGCCTGTGGG	GGAGTACCAG	GATGTGCCTG	ACCTGGTCTG	GAGCCTCGAT	1560
GGCATTCCCT	TCCGTGGTGG	CCCCACCCTG	ACGGGCAGTG	CCTTGCGGCA	GGCGGCAGAG	1620
CGTGGCTTCG	GGAGCGCCAC	CAGGACAGGC	CAGGACCGGC	CACGTAGAGT	GGTGGTTTTG	1680
CTCACTGAGT	CACACTCCGA	GGATGAGGTT	GCGGGCCCAG	CGCGTCACGC	AAGGGCGCGA	1740
GAGCTGCTCC	TGCTGGGTGT	AGGCAGTGAG	GCCGTGCGGG	CAGAGCTGGA	GGAGATCACA	1800
GGCAGCCCAA	AGCATGTGAT	GGTCTACTCG	GATCCTCAGG	ATCTGTTCAA	CCAAATCCCT	1860
GAGCTGCAGG	GGAAGCTGTG	CAGCCGGCAG	CGGCCAGGGT	GCCGGACACA	AGCCCTGGAC	1920
CTCGTCTTCA	TGTTGGACAC	CTCTGCCTCA	GTAGGGCCCG	AGAATTTTGC	TCAGATGCAG	1980
AGCTTTGTGA	GAAGCTGTGC	CCTCCAGTTT	GAGGTGAACC	CTGACGTGAC	ACAGGTCCGG	2040
CTGGTGGTGT	ATGGCAGCCA	GGTGCACT	GCCTTCGGGC	TGGACACCAA	ACCCACCCGG	2100
GCTGCGATGC	TGCGGGCCAT	TAGCCAGGCC	CCCTACCTAG	GTGGGTGGG	CTCAGCCGGC	2160
ACCGCCCTGC	TGCACATCTA	TGACAAAGTG	ATGACCGTCC	AGAGGGGTGC	CCGGCCTGCT	2220
GTCCCCAAAG	CTGTGGTGGT	GCTCACAGGC	GGGAGAGGGC	CAGAGGATGC	AGCCGTTCCCT	2280
GCCCAGAAGC	TGAGTAACAA	TGGCATCTCT	GCTTGGTTCG	TGGGCGTGGG	GCCTGTCCCTA	2340
AGTGAGGGTC	TGCGGAGGCT	TGCAGGTCCC	CGGGATTCCC	TGATCCACGT	GGCAGCTTAC	2400
GCCGACCTGC	GGTACCACCA	GGACGTGCTC	ATTGAGTGGC	TGTGTGGAGA	AGCCAAGCAG	2460
CCAGTCAACC	TCTGCAAAACC	CAGCCCCTGC	ATGAATGAGG	GCAGCTGCGT	CCTGCAGAAAT	2520
GGGAGCTACC	GCTGCAAGTG	TCGGGATGGC	TGGGAGGGGC	CCCCTGCGA	GAACCGTGAG	2580
TGGAGCTCTT	GCTCTGTATG	TGTGAGCCAG	GGATGGATTG	TTGAGACGCC	CCTGAGGCAC	2640
ATGGCTCCCG	TGCAGGAGGG	CAGCAGCCGT	ACCCCTCCCA	GCAACTACAG	AGAAGGCCTG	2700
GGCACTGAAA	TGGTGCCTAC	CTTCTGGAAT	GTCTGTGCCC	CAGGTCCTTA	GAATGTCTGC	2760
TTCCCGCCGT	GGCCAGGACC	ACTATTCTCA	CTGAGGGAGG	AGGATGTCCC	AACTGCAGCC	2820

ATGCTGCTTA	GAGACAAGAA	AGCAGCTGAT	GTCACCCACA	AACGATGTTG	TTGAAAAGTT	2880
TTGATGTGTA	AGTAAATACC	CACTTCTGT	ACCTGCTGTG	CCTTGTTGAG	GCTATGTCAT	2940
CTGCCACCTT	TCCCTTGAGG	ATAAACAAAG	GGTCCTGAAG	ACTTAAATTT	AGCGGCCTGA	3000
CGTTCCTTTG	CACACAATCA	ATGCTCGCCA	GAATGTTGTT	GACACAGTAA	TGCCCAGCAG	3060
AGGCCTTTAC	TAGAGCATCC	TTTGGACGGC	GAAGGCCACG	GCCTTTC AAG	ATGGAAAGCA	3120
GCAGCTTTTC	CACTTCCCCA	GAGACATTCT	GGATGCATTT	GCATTGAGTC	TGAAAGGGGG	3180
CTTGAGGGAC	GTTTGTGACT	TCTTGGCGAC	TGCCTTTTGT	GTGTGGAAGA	GACTTGAAAA	3240
GGTCTCAGAC	TGAATGTGAC	CAATTAACCA	GCTTGGTTGA	TGATGGGGGA	GGGGCTGAGT	3300
TGTGCATGGG	CCCAGGTCTG	GAGGGCCACG	TAAAATCGTT	CTGAGTCGTG	AGCAGTGTCC	3360
ACCTTGAAGG	TCTTC					

### CBF9 Protein sequence (SEQ ID NO: 2)

Gene name: ESTs  
Unigene number: Hs.157601

Protein Accession #: none found

Signal sequence: 1-17  
Transmembrane domains: none found  
VGW domains: 49-223; 341-518; 529-706  
EGF domains: 298-333; 715-748  
Cellular Localization: plasma membrane

1	11	21	31	41	51	
MPPFLLLEAV	CVFLFSRVPP	SLPLQEVHVS	KETIGKISAA	SKMMWCSAAV	DIMFLLDGSN	60
SVGKGSFERS	KHFAITVCDG	LDISPERVRV	GAQFSSTPH	LEFPLDSFST	QDEVKARIKR	120
MVFKGGRTET	ELALKYLLHR	GLPGGRNASV	PQILIIIVTDG	KSQGDVALPS	KQLKERGVTV	180
FAVGVRFPWR	EELHALASEP	RGQHVLLAEQ	VEDATNGLFS	TLSSSAICSS	ATPDCRVEAH	240
PCEHRTLEMV	REFAGNAPCW	RGSRRTLAVL	AAHCPFYSWK	RVFLTHPATC	YRTTCPGPCD	300
SQPCQNGGTC	VPEGLDGYQC	LCPLAFGGEA	NCALKLSLEC	RVDLLFLLDS	SAGTTLDGFL	360
RAKVFKRFV	RAVLSEDSRA	RVGVATYSRE	LLVAVPVGEY	QDVPDLVWSL	DGIPFRGGPT	420
LTGSALRQAA	ERGFSGSATRT	GQDRPRRVVV	LLTESHSEDE	VAGPARHARA	RELLLLGVGS	480
EAVRAELEEI	TGSPKHMVMY	SDPQDLFNQI	PELQGKLC SR	QRP GCRTQAL	DLVFMLDTSA	540
SVGPENFAQM	QS FVRSCALQ	FEVNP DVTQV	GLVVYGSQVQ	TAFGLDTKPT	RAAMLRAISQ	600
APYLGGVGSA	GTALLHIYDK	VMTVQRGARP	GVPKAVVVL T	GGRGAEDAAV	PAQKL RNNGI	660
SVLVVGVPV	LSEGLRRLAG	PRDSLIHVAA	YADLRYHQDV	LIEWLCGEAK	QPVNLCKPSP	720
CMNEGSCVLQ	NGSYRCKCRD	GWEGPHCENR	EWSSCSVCVS	QGWILETPLR	HMAPVQEGSS	780
RTPPSNYREG	LGTEMVPTFW	NVCAPGP				

### CVA7 DNA and Protein Sequences

#### CVA7 DNA sequence (SEQ ID NO: 3)

Nucleic Acid Accession #: XM\_051860.2  
Coding sequence: 52..3042

1	11	21	31	41	51	
GCTCACCCAG	GAAAAATATG	CAATCGTCCC	ATTGATATAC	AGGCCACTAC	AATGGATGGA	60
GTTAACCTCA	GCACCGAGGT	TGTCTACAAA	AAAGGCCAGG	ATTATAGGTT	TGCTTGCTAC	120
GACCGGGGCA	GAGCCTGCCG	GAGCTACCGT	GTACGGTTCC	TCTGTGGGAA	GCCTGTGAGG	180
CCCAAAC TCA	CAGTCACCAT	TGACACCAAT	GTGAACAGCA	CCATTCTGAA	CTTGGAGGAT	240
AATGTACAGT	CATGGAAACC	TGGAGATACC	CTGGTCATTG	CCAGTACTGA	TTACTCCATG	300
TACCAGGCAG	AAGAGTTCCA	GGTGCTTCCC	TGCAGATCCT	GCGCCCCCAA	CCAGGTCAAA	360
GTGGCAGGGA	AACCAATTGA	CCTGCACATC	GGGGAGGAGA	TAGACGGCGT	GGACATGCCG	420
GCGGAGGTTG	GGCTTCTGAG	CCGGAACATC	ATAGTGATGG	GGGAGATGGA	GGACAAATGC	480
TACCCCTACA	GAAACCACAT	CTGCAATTTT	TTTGACTTCG	ATACCTTTGG	GGGCCACATC	540
AAGTTTGCTC	TGGGATT TAA	GGCAGCACAC	TTGGAGGGCA	CGGAGCTGAA	GCATATGGGA	600
CAGCAGCTGG	TGGGTCAGTA	CCC GATTAC	TTCCACCTGG	CCGGTGATGT	AGACGAAAGG	660
GGAGGTTATG	ACCCACCCAC	ATACATCAGG	GACCTCTCCA	TCCATCATAC	ATTCTCTCGC	720
TGCGTCACAG	TCCATGGCTC	CAATGGCTTG	TTGATCAAGG	ACGTTGTGGG	CTATAACTCT	780
TTGGGCCACT	GCTTCTTCAC	GGAAGATGGG	CCGGAGGAAC	GCAACACTTT	TGACCACTGT	840
CTTGGCCTCC	TTGTCAAGTC	TGGAACCCTC	CTCCCCCTCG	ACCGTGACAG	CAAGATGTGC	900
AAGATGATCA	CAGGAGACTC	CTACCCAGGG	TACATCCCCA	AGCCCAGGCA	AGACTGCAAT	960



GCTGTGTCCA	CCTTCTGGAT	GGCCAATCCC	AACAACAACC	TCATCAACTG	TGCCGCTGCA	1020
GGATCTGAGG	AAACTGGATT	TTGGTTTATT	TTTCACCACG	TACCAACGGG	CCCCTCCGTG	1080
GGAAATGTACT	CCCCAGGTTA	TTTCAGAGCAC	ATTCCACTGG	GAAAATTCTA	TAACAACCGA	1140
GCACATTCCA	ACTACCGGGC	TGGCATGATC	ATAGACAACG	GAGTCAAAAC	CACCGAGGCC	1200
TCTGCCAAGG	ACAAGCGGCC	GTTCTCTCTA	ATCATCTCTG	CCAGATACAG	CCCTCACCAG	1260
GACGCCGACC	CGCTGAAGCC	CCGGGAGCCG	GCCATCATCA	GACACTTCAT	TGCCTACAAG	1320
AACCAGGACC	ACGGGGCCTG	GCTGCGCGGC	GGGGATGTGT	GGCTGGACAG	CTGCCGGTTT	1380
GCTGACAATG	GCATTGGCCT	GACCCCTGGC	AGTGGTGGAA	CCTTCCCCTA	TGACGACGGC	1440
TCCAAGCAAG	AGATAAAGAA	CAGCTTGTTT	GTTGGCGAGA	GTGGCAACGT	GGGGACGGAA	1500
ATGATGGACA	ATAGGATCTG	GGGCCCTGGC	GGCTTGGAAC	ATAGCGGAAG	GACCCCTCCCT	1560
ATAGGCCAGA	ATTTTCCAAT	TAGAGGAATT	CAGTTATATG	ATGGCCCCAT	CAACATCCAA	1620
AACTGCACAT	TCCGAAAGTT	TGTGGCCCTG	GAGGGCCGGC	ACACCAGCGC	CCTGGCCTTC	1680
CGCTTGAATA	GATGCCTGGC	GAGTGCCTCC	CATAACAACG	TGACCGGCAT	TGCCTTTGAG	1740
GACGTTCCGA	TTACTTCCAG	AGTGTCTTTC	GGAGAGCCTG	GGCCCTGGTT	CAACCAGCTG	1800
GACATGGATG	GGGATAAGAC	ATCTGTGTTC	CATGACGTGC	ACGGCTCCGT	GTCCGAGTAC	1860
CCTGGCTCCT	ACCTCACGAA	GAATGACAAC	TGGCTGGTCC	GGCACCAGCA	CTGCATCAAT	1920
GTTCCCGACT	GGAGAGGGGC	CATTTGCAGT	GGGTGCTATG	CACAGATGTA	CATTCAAGCC	1980
TACAAGACCA	GTAACCTGCG	AATGAAGATC	ATCAAGAATG	ACTTCCCCAG	CCACCCCTTT	2040
TACCTGGAGG	GGGCGCTCAC	CAGGAGCACC	CATTACCAGC	AATACCAACC	GGTTGTCAAC	2100
CTGCAGAAGG	GCTACACCAT	CCACTGGGAC	CAGACGGCCC	CCGCCGAAC	CGCCATCTGG	2160
CTCATCAACT	TCAACAAGGG	CGACTGGATC	CGAGTGGGGC	TCTGCTACCC	GCGAGGCACC	2220
ACATTTCTCA	TCCTCTCGGA	TGTTTACAAT	CGCTGTCTGA	AGCAAACGTC	CAAGACGGGC	2280
TCTTTCGTGA	GGACCTTGCA	GATGGACAAA	GTGGAGCAGA	GCTACCCTGG	CAGGAGCCAC	2340
TACTACTGGG	ACGAGGACTC	AGGGCTGTTG	TTCTTGAAGC	TGAAAGCTCA	GAACGAGAGA	2400
GAGAAGTTTG	CTTTCTGCTC	CATGAAAGGC	TGTGAGAGGA	TAAAGATTAA	AGCTCTGATT	2460
CCAAAGAAGC	CAGGCGTCAG	TGACTGCACA	GCCACAGCTT	ACCCCAAGTT	CACCGAGAGG	2520
GCTGTCTGTG	ACGTGCCGAT	GCCCAAGAAG	CTCTTTGGTT	CTCAGCTGAA	AACAAAGGAC	2580
CATTTCTTGG	AGGTGAAGAT	GGAGAGTTCC	AAGCAGCACT	TCTTCCACCT	CTGGAACGAC	2640
TTTCGCTTACA	TTGAAGTGGA	TGGGAAGAAG	TACCCCAAGT	CGGAGGATGG	CATCCAGGTG	2700
GTGGTGATTG	ACGGGAACCA	AGGGCGCGTG	GTGAGCCACA	CGAGCTTCAG	GAACCTCCATT	2760
CTGCAAGGCA	TACCATGGCA	GCTTTTCAAC	TATGTGGCGA	CCATCCCTGA	CAATTCCATA	2820
GTGCTTATGG	CATCAAAGGG	AAGATACGTC	TCCAGAGGCC	CATGGACCAG	AGTGTCTGAA	2880
AAGCTTGGGG	CAGACAGGGG	TCTCAAGTTG	AAAGAGCAAA	TGGCATTCGT	TGGCTTCAAA	2940
GGCAGCTTCC	GGCCCATCTG	GGTGACACTG	GACACTGAGG	ATCACAAAGC	CAAAATCTTC	3000
CAAGTTGTGC	CCATCCCTGT	GGTGAAGAAG	AAGAAGTTGT	GAGGACAGCT	GCCGCCCCGT	3060
GCCACCTCGT	GGTAGACTAT	GACGGTGAAT	CTTGGCAGCA	GACCACTGGG	GGATGGCTGG	3120
GTCCCCCAGC	CCCTGCCAGC	AGCTGCCTGG	GAAGGCCCGT	TTTCAGCCCT	GATGGGCCAA	3180
GGGAAGGCTA	TCAGAGACCC	TGGTGCTGCC	ACCTGCCCC	ACTCAAGTGT	CTACCTGGAG	3240
CCCTTGGGGC	GGTGCTGGCC	AATGCTGGAA	ACATTCACTT	TCCTGCAGCC	TCTTGGGTGC	3300
TTCTCTCCTA	TCTGTGCCTC	TTCAGTGGGG	GTTTGGGGAC	CATATCAGGA	GACCTGGGTT	3360
GTGCTGACAG	CAAAGATCCA	CTTTGGCAGG	AGCCCTGACC	CAGCTAGGAG	GTAGTCTGGA	3420
GGGCTGGTCA	TTCACAGATC	CCCATGGTCT	TCAGCAGACA	AGTGAGGGTG	GTAAATGTAG	3480
GAGAAAGAGC	CTTGGCCCTT	AGGAAATCTT	TACTCCTGTA	AGCAAGAGCC	AACCTCACAG	3540
GATTAGGAGC	TGGGGTAGAA	CTGGCTATCC	TTGGGGGAAGA	GGCAAGCCCT	GCCTCTGGCC	3600
GTGTCCACCT	TTCAGGAGAC	TTTGAGTGGC	AGGTTTGGAC	TTGGACTAGA	TGACTCTCAA	3660
AGGCCCTTTT	AGTTCTGAGA	TTCCAGAAAT	CTGTCTGCAT	TCACATGGTA	CCTGGAACCC	3720
AACAGTTCAT	GGATATCCAC	TGATATCCAT	GATGTGGGT	GCCCCAGCGC	ACACGGGATG	3780
GAGAGGTGAG	AACATAATGCC	TAGCTTGAGG	GGTCTGCAGT	CCAGTAGGGC	AGGCAGTCAG	3840
TGCCATGTGC	ACTGCAATGC	CAGGTGGAGA	AATCACAGAG	AGGTAAAATG	GAGGCCAGTG	3900
CCATTTTACA	GGGGAGGCTC	AGGAAGGCTT	CTTGCTTACA	GGAATGAAGG	CTGGGGGCAT	3960
TTTGCTGGGG	GGAGATGAGG	CAGCCTCTGG	AATGGCTCAG	GGATTACAGC	CTCCCTGCCG	4020
CTGCCTGCTG	AAGCTGGTGA	CTACGGGGTC	GCCCTTTGCT	CACGTCTCTC	TGGCCCACTC	4080
ATGATGGAGA	AGTGTGGTCA	GAGGGGAGCA	ATGGGCTTTG	CTGCTTATGA	GCACAGAGGA	4140
ATTCAGTCCC	CAGGCAGCCC	TGCCTCTGAC	TCCAAGAGGG	TGAAGTCCAC	AGAAGTGAGC	4200
TCCTGCCTTA	GGGCCTCATT	TGCTCTTCAT	CCAGGGAAC	GAGCACAGGG	GGCCTCCAGG	4260
AGACCCTAGA	TGTGCTCGTA	CTCCCTCGGC	CTGGGATTTT	AGAGCTGGAA	ATATAGAAAA	4320
TATCTAGCCC	AAAGCCTTCA	TTTAAACAGA	TGGGGAAAGT	GAGCCCCCAA	GATGGGAAAG	4380
AACCAACAG	CTAAGGGAGG	GCCTGGGGAG	CCCCACCCTA	GCCCTTGCTG	CCACACCACA	4440
TTCCTTCAAC	AACCGGCCCC	AGAGTGCCCC	GGCACTCCTG	AGGTAGCTTC	TGGAAATGGG	4500
GACAAGTCCC	CTCGAAGGAA	AGGAAATGAC	TAGAGTAGAA	TGACAGCTAG	CAGATCTCTT	4560
CCCTCCTGCT	CCCAGCGCAC	ACAAACCCGC	CCTCCCCTTG	GTGTTGGCGG	TCCCTGTGGC	4620
CTTCACTTTG	TTCACTACCT	GTCAGCCACG	CCTGGGTGCA	CAGTAGCTGC	AACTCCCCAT	4680
TGGTGTCTAC	TGGCTCTCCT	GTCTCTGCAG	CTCTACAGGT	GAGGCCCAGC	AGAGGGAGTA	4740
GGGCTCGCCA	TGTTTCTGGT	GAGCCAATTT	GGCTGATCTT	GGGTGTCTGA	ACAGCTATTG	4800
GGTCCACCCC	AGTCCCTTTT	AGCTGCTGCT	TAATGCCCTG	CTCTCTCCCT	GGCCACCTTT	4860
ATAGAGAGCC	CAAAGAGCTC	CTGTAAGAGG	GAGAACTCTA	TCTGTGGTTT	ATAATCTTGC	4920
ACGAGGCACC	AGAGTCTCCC	TGGGTCTTGT	GATGAACTAC	ATTTATCCCC	TTTCTGCCCC	4980
CAACCACAAA	CTCTTTCTCT	CAAAGAGGGC	CTGCCTGGCT	CCCTCCACCC	AACTGCACCC	5040

ATGAGACTCG	GTCCAAGAGT	CCATTCCCCA	GGTGGGAGCC	AACTGTCAGG	GAGGTCTTTT	5100
CCACCAAACA	TCTTTCAGCT	GCTGGGAGGT	GACCATAGGG	CTCTGCTTTT	AAAGATATGG	5160
CTGCTTCAAA	GGCCAGAGTC	ACAGGAAGGA	CTTCTTCCAG	GGAGATTAGT	GGTGATGGAG	5220
AGGAGAGTTA	AAATGACCTC	ATGTCCTTCT	TGTCCACGGT	TTTGTTGAGT	TTTCACTCTT	5280
CTAATGCAAG	GGTCTCACAC	TGTGAACCAC	TTAGGATGTG	ATCACTTTCA	GGTGGCCAGG	5340
AATGTTGAAT	GTCTTTGGCT	CAGTTCATTT	AAAAAAGATA	TCTATTTGAA	AGTTCTCAGA	5400
GTTGTACATA	TGTTTCACAG	TACAGGATCT	GTACATAAAA	GTTTCTTTCC	TAAACCATTTC	5460
ACCAAGAGCC	AATATCTAGG	CATTTTCTTG	GTAGCACAAA	TTTTCTTATT	GCTTAGAAAA	5520
TTGTCTCCT	TGTTATTTCT	GTTTGTAAAG	CTTAAGTGAG	TTAGGTCTTT	AAGGAAAGCA	5580
ACGCTCCTCT	GAAATGCTTG	TCTTTTTTCT	GTTGCCGAAA	TAGCTGGTCC	TTTTTCGGGA	5640
GTTAGATGTA	TAGAGTGTTT	GTATGTAAAC	ATTTCTTGTA	GGCATCACCA	TGAACAAAGA	5700
TATATTTTCT	ATTTATTTAT	TATATGTGCA	CTTCAAGAAG	TCAGTGTGAG	AGAAATAAAG	5760
AATTGTCTTA	AATGTCAAAA	AAAAAAAAAA	AAAAAAAAAA	AAAAAAAAAA		

# CVA7 Protein sequence (SEQ ID NO: 4)

Protein Accession #: XP\_051860.2

1	11	21	31	41	51	
MDGVNLSTEV	VYKKGQDYRF	ACYDRGRACR	SYRVRFLCGK	PVRPKLTVTI	DTNVNSTILN	60
LEDNVQSWKP	GDTLVIASTD	YSMYQAEFQ	VLPCRSCAPN	QVKVAGKPMY	LHIGEEIDGV	120
DMRAEVGLLS	RNIIVMGEME	DKCYPYRNHI	CNFFDFDTFG	GHIKFALGFK	AAHLEGTELK	180
HMGQQLVGQY	PIHFHLAGDV	DERGGYDPPT	YIRDLSIHHT	FSRCVTVHGS	NGLLIKDVVG	240
YNSLGHCFTF	EDGPEERNFT	DHCLGLLVKS	GTLPLPSDRDS	KMCKMITGDS	YPGYIPKPRQ	300
DCNAVSTFWM	ANPNNNLINC	AAAGSEETGF	WFI FHHVPTG	PSVGMYSPTY	SEHIPLGKFY	360
NNRAHSNYRA	GMIIDNGVKT	TEASAKDKRP	FLSIISARYS	PHQDADPLKP	REPAIRRHFI	420
AYKNQDHGAW	LRGGDVWLDS	CRFADNGIGL	TLASGGTFPY	DDGSKQEIKN	SLFVGESGNV	480
GTEMDNRIW	GPGLLDHSGR	TLPIGQNFPI	RGIQLYDGPI	NIQNCTFRKF	VALEGRHTSA	540
LAFLRLNAWQ	SCPHNNVTGI	AFEDVPITSR	VFFGEPGPWF	NQLDMDGDKT	SVFHDVDGSV	600
SEYPGSYLTK	NDNWLVRHPD	CINVPDWRGA	ICSGCYAQMY	IQAYKTSNLR	MKIIKNDFPS	660
HPLYLEGALT	RSTHYQQYQP	VVTLQKGYTI	HWDQTAPAEI	AIWLINFNKG	DWIRVGLCYP	720
RGTTFSILSD	VHNRLLKQTS	KTGVFVRTLQ	MDKVEQSYPG	RSHYYWDEDS	GLLFLKLKAQ	780
NEREKFAFCS	MKGCEIRIKI	ALIPKNAGVS	DCTATAYPKF	TERAVVDVPM	PKKLFGSQLK	840
TKDHFLEVKM	BSSKQHFFHL	WNDFAYIEVD	GKKYPSSEDG	IQVVVIDGNQ	GRVVSHTSFR	900
NSILQGIPIW	LFNYVATIPD	NSIVLMASKG	RYVSRGPWTR	VLEKLGADRG	LKLKEQMAFV	960
GFKGSFRPIW	VTLDTEDHKA	KIFQVVPPIV	VKKKKL			

# CVA7 variant DNA sequence (SEQ ID NO: 5)

Nucleic Acid Accession #: Eos sequence

Coding sequence: 261..2861

1	11	21	31	41	51	
GAGCTAGCGC	TCAAGCAGAG	CCCAGCGCGG	TGCTATCGGA	CAGAGCCTGG	CGAGCGCAAG	60
CGGCGCGGGG	AGCCAGCGGG	GCTGAGCGCG	GCCAGGGTCT	GAACCCAGAT	TTCCCAGACT	120
AGCTACCACT	CCGCTTGCCC	ACGCCCCGGG	AGCTCGCGGC	GCCTGGCGGT	CAGCGACCAG	180
ACGTCCGGGG	CCGCTGCGCT	CCTGGCCCCG	GAGGCGTGAC	ACTGTCTCGG	CTACAGACCC	240
AGAGGGAGCA	CACTGCCAGG	ATGGGAGCTG	CTGGGAGGCA	GGACTTCCTC	TTCAAGGCCA	300
TGCTGACCAT	CAGCTGGCTC	ACTCTGACCT	GCTTCCCTGG	GGCCACATCC	ACAGTGGCTG	360
CTGGGTGCCC	TGACCAGAGC	CCTGAGTTGC	AACCTTGGAA	CCCTGGCCAT	GACCAAGACC	420
ACCATGTGCA	TATCGGCCAG	GGCAAGACAC	TGCTGCTCAC	CTCTTCTGCC	ACGGTCTATT	480
CCATCCACAT	CTCAGAGGGA	GGCAAGCTGG	TCATTAAAGA	CCACGACGAG	CCGATTGTTT	540
TGCGAACCCG	GCACATCCTG	ATTGACAACG	GAGGAGAGCT	GCATGCTGGG	AGTGCCCTCT	600
GCCCTTTCCA	GGGCAATTTT	ACCATCATTT	TGTATGGAAG	GGCTGATGAA	GGTATTGAGC	660
CGGATCCTTA	CTATGGTCTG	AAGTACATTG	GGGTTGGTAA	AGGAGGCGCT	CTTGAGTTGC	720
ATGGACAGAA	AAAGCTCTCC	TGGACATTTT	TGAACAAGAC	CCTTCACCCA	GGTGGCATGG	780
CAGAAGGAGG	CTATTTTTTT	GAAAGGAGCT	GGGGCCACCG	TGGAGTTATT	GTTTCATGTCA	840
TCGACCCCAA	ATCAGGCACA	GTATCCATT	CTGACCGGTT	TGACACCTAT	AGATCCAAGA	900
AAGAGAGTGA	ACGTCTGGTC	CAGTATTTGA	ACGCGGTGCC	CGATGGCAGG	ATCCTTTCTG	960
TTCAGTGAA	TGATGAAGGT	TCTCGAAATC	TGGATGACAT	GGCCAGGAAG	GCGATGACCA	1020
AATTGGGAAG	CAAACTCTTC	CTGCACCTTG	GATTTAGACA	CCCTTGGAGT	TTTCTAACTG	1080
TGAAAGGAAA	TCCATCATCT	TCAGTGAAG	ACCATATTGA	ATATCATGGA	CATCGAGGCT	1140
CTGCTGCTGC	CCGGGTATTC	AAATTGTTCC	AGACAGAGCA	TGGCGAATAT	TTCAATGTTT	1200
CTTTGTCCAG	TGAGTGGGTT	CAAGACGTGG	AGTGGACGGA	GTGGTTTCGAT	CATGATAAAG	1260
TATCTCAGAC	TAAAGGTGGG	GAGAAAATTT	CAGACCTCTG	GAAAGCTCAC	CCAGGAAAAA	1320
TATGCAATCG	TCCCAATTGAT	ATACAGGCCA	CTACAATGGA	TGGAGTTAAC	CTCAGCACCG	1380
AGGTTGTCTA	CAAAAAAGGC	CAGGATTATA	GGTTTGCTTG	CTACGACCGG	GGCAGAGCCT	1440

GCCGGAGCTA	CCGTGTACGG	TTCCTCTGTG	GGAAGCCTGT	GAGGCCCAAA	CTCACAGTCA	1500
CCATTGACAC	CAATGTGAAC	AGCACCATTC	TGAACCTTGA	GGATAATGTA	CAGTCATGGA	1560
AACCTGGAGA	TACCCCTGGTC	ATTGCCAGTA	CTGATTACTC	CATGTACCAG	GCAGAAGAGT	1620
TCCAGGTGCT	TCCCTGCAGA	TCCTGCGCCC	CCAACCAGGT	CAAAGTGGCA	GGGAAACCAA	1680
TGTACCTGCA	CATCGGGGAG	GAGATAGACG	GCGTGGACAT	GCGGGCGGAG	GTTGGGCTTC	1740
TGAGCCGGAA	CATCATAGTG	ATGGGGGAGA	TGGAGGACAA	ATGCTACCCC	TACAGAAACC	1800
ACATCTGCAA	TTTCTTTGAC	TTCGATACCT	TTGGGGGGCCA	CATCAAGTTT	GCTCTGGGAT	1860
TTAAGGCAGC	ACACTTGGAG	GGCACGGAGC	TGAAGCATAT	GGGACAGCAG	CTGGTGGGTC	1920
AGTACCCGAT	TCACCTCCAC	CTGGCCGGTG	ATGTAGACGA	AAGGGGAGGT	TATGACCCAC	1980
CCACATACAT	CAGGGACCTC	TCCATCCATC	ATACATTCTC	TCGCTGCGTC	ACAGTCCATG	2040
GCTCCAATGG	CTTGTTGATC	AAGGACGTTG	TGGGCTATAA	CTCTTTGGGC	CACTGCTTCT	2100
TCACGGAAGA	TGGGCCGGAG	GAACGCAACA	CTTTTGACCA	CTGTCTTGCC	CTCCTTGTC	2160
AGTCTGGAAC	CCTCCTCCCC	TCGGACCGTG	ACAGCAAGAT	GTGCAAGATG	ATCACAGAGG	2220
ACTCCTACCC	AGGGTACATC	CCCAAGCCCA	GGCAAGACTG	CAATGCTGTG	TCCACCTTCT	2280
GGATGGCCAA	TCCCAACAAC	AACCTCATCA	ACTGTGCCGC	TGCAGGATCT	GAGGAACTG	2340
GATTTTGGTT	TATTTTTCAC	CACGTACCAA	CGGGCCCCCTC	CGTGGGAATG	TACTCCCCAG	2400
GTTATTGAGA	GCACATTCCA	CTGGGAAAAT	TCTATAACAA	CCGAGCACAT	TCCAACCTACC	2460
GGGCTGGCAT	GATCATAGAC	AACGGAGTCA	AAACCACCGA	GGCCTCTGCC	AAGGACAAGC	2520
GGCCGTTCTT	CTCAATCATC	TCTGCCAGAT	ACAGCCCTCA	CCAGGACGCC	GACCCGCTGA	2580
AGCCCCGGGA	GCCGGCCATC	ATCAGACACT	TCATTGCCTA	CAAGAACCAG	GACCACGGGG	2640
CCTGGCTGCG	CGGCGGGGAT	GTGTGGCTGG	ACAGCTGCCA	TTTCAGAGGG	GAGGCTCAGG	2700
AAGGCTTCTT	GCTTACAGGA	ATGAAGGCTG	GGGGCATTTT	GCTGGGGGGA	GATGAGGCAG	2760
CCTCTGGAAT	GGCTCAGGGA	TTCAGCCCTC	CCTGCCGTG	CCTGCTGAAG	CTGGTGACTA	2820
CGGGTTCGCC	CTTTGCTCAC	GTCTCTCTGG	CCCACTCATG	ATGGAGAAGT	GTGGTCAGAG	2880
GGGAGCAATG	GGCTTTGCTG	CTTATGAGCA	CAGAGGAATT	CAGTCCCCAG	GCAGCCCTGC	2940
CTCTGACTCC	AAGAGGGTGA	AGTCCACAGA	AGTGAGCTCC	TGCCTTAGGG	CCTCATTTGC	3000
TCTTCATCCA	GGGAACCTGAG	CACAGGGGGC	CTCCAGGAGA	CCCTAGATGT	GCTCGTACTC	3060
CCTCGGCCTG	GGATTTTCTG	GCTGGAAATAT	TAGAAAATAT	CTAGCCCCAA	GCCTTCATTT	3120
TAACAGATGG	GGAAAGTGAG	CCCCAAGAT	GGGAAAGAAC	CACACAGCTA	AGGGAGGGCC	3180
TGGGGAGCCC	CACCCTAGCC	CTTGCTGCCA	CACCACATTG	CCTCAACAAC	CGGCCCCAGA	3240
GTGCCCAGGC	ACTCCTGAGG	TAGCTTCTGG	AAATGGGGAC	AAGTCCCCCTC	GAAGGAAAGG	3300
AAATGACTAG	AGTAGAATGA	CAGCTAGCAG	ATCTCTTCCC	TCCTGCTCCC	AGCGCACACA	3360
AACCCGCCCT	CCCCTTGGTG	TTGGCGGTCC	CTGTGGCCTT	CACCTTGCTC	ACTACCTGTC	3420
AGCCAGCCCT	GGGTGCACAG	TAGCTGCAAC	TCCCCATTGG	TGCTACCTGG	CTCTCTGTGC	3480
TCTGCAGCTC	TACAGGTGAG	GCCCAGCAGA	GGGAGTAGGG	CTCGCCATGT	TTCTGGTGAG	3540
CCAATTTGGC	TGATCTTGGG	TGTCTGAACA	GCTATTGGGT	CCACCCCACT	CCCTTTTCAGC	3600
TGCTGCTTAA	TGCCCTGCTC	TCTCCCTGGC	CCACCTTATA	GAGAGCCCAA	AGAGCTCCTG	3660
TAAGAGGGAG	AACTCTATCT	GTGGTTTATA	ATCTTGCACG	AGGCACCAGA	GTCTCCCTGG	3720
GTCTTGTGAT	GAACCTACAT	TATCCCCTTT	CCTGCCCCAA	CCACAACTC	TTTCCTTCAA	3780
AGAGGGCCTG	CCTGGCTCCC	TCCACCCAAC	TGCACCCATG	AGACTCGGTC	CAAGAGTCCA	3840
TTCCCCAGGT	GGGAGCCAAC	TGTCAGGGAG	GTCTTTCCCA	CCAAACATCT	TTCAGCTGCT	3900
GGGAGGTGAC	CATAGGGCTC	TGCTTTTAAA	GATATGGCTG	CTTCAAAGGC	CAGAGTCACA	3960
GGAAGGACTT	CTTCCAGGGA	GATTAGTGTT	GATGGAGAGG	AGAGTTAAAA	TGACCTCATG	4020
TCTTCTTGT	CCACGGTTTT	GTTGAGTTTT	CACCTTCTTA	ATGCAAGGGT	CTCACACTGT	4080
GAACCACTTA	GGATGTGATC	ACTTTCAGGT	GGCCAGGAAT	GTTGAATGTC	TTTGGCTCAG	4140
TTCATTTAAA	AAAGATATCT	ATTGAAAGT	TCTCAGAGTT	GTACATATGT	TTCACAGTAC	4200
AGGATCTGTA	CATAAAAGTT	TCTTTCCTAA	ACCATTACCC	AAGAGCCAAAT	ATCTAGGCAT	4260
TTTCTTGGTA	GCACAAATTT	TCTTATTGCT	TAGAAAATTG	TCCTCCTTGT	TATTTCTGTT	4320
TGTAAGACTT	AAGTGAGTTA	GGTCTTTAAG	GAAAGCAACG	CTCCTCTGAA	ATGCTTGTCT	4380
TTTTTCTGTT	GCCGAAATAG	CTGGTCTTTT	TTCGGGAGTT	AGATGTATAG	AGTGTTTGTA	4440
TGTAAACATT	TCTGTAGGC	ATCACCATGA	ACAAAGATAT	ATTTTCTATT	TATTTATTAT	4500
ATGTGCACTT	CAAGAAGTCA	CTGTCAGAGA	AATAAAGAAT	TGTCTTAAAT	GTCATGATTG	4560
GAGATGTCCT	TTGCATTGCT	TGGAAGGGGT	GTACCTAGAG	CCAAGGAAAT	TGGCTCTGGT	4620
TTGGAAAAAT	TTTGCTGTTA	TTATAGTAAA	CATACAAAGG	ATGTCAAAAA	AAAAAATAAA	4680
AAAAAATAAA	AAAAAATAAA	AA				

# CVA7 variant Protein sequence (SEQ ID NO: 6)

Protein Accession #: Eos sequence

1	11	21	31	41	51	
MGAAGRQDFL	FKAMLTISWL	TLTCFPGATS	TVAAGCPDQS	PELQPNWPGH	DQDHHVHIGQ	60
GKTLTLLTSSA	TVYSIHISEG	GKLVIKDHDE	PIVLRTRHIL	IDNGGELHAG	SALCPFQGNF	120
TIILYGRADE	GIQPDPPYGL	KYIGVGKGGG	LELHGQKKLS	WTFLNKTLP	GGMAEGGYFF	180
ERSWGHHRGI	VHVIDPKSGT	VIHSDRFDTY	RSKKESERLV	QYLNVAVPDGR	ILSVAVNDEG	240
SRNLDDMARK	AMTKLGSKHF	LHLGFRHPWS	FLTVKGNPSS	SVEDHIEYHG	HRGSAAARVF	300
KLFQTEHGEY	FNVSLSSEWV	QDVEWTEWFD	HDKVSQTKGG	EKISDLWKAH	PGKICNRPID	360
IQATTMDGVN	LSTEVVYKKG	QDYRFACYDR	GRACRSYVR	FLCGKPVVRK	LTVTIDTNVN	420

STILNLEDNV	QSWKPGDTLV	IASTDYSMYQ	AEEFQVLPCR	SCAPNQVKVA	GKPMYLHIGE	480
EIDGVDMRAE	VGLLSRNIIV	MGEMEDKCYP	YRNHICNFFD	FDTFGGHIKF	ALGFKAAHLE	540
GTELKHMGGQ	LVGQYPIHFH	LAGDVDERGG	YDPPTYIRDL	SIHHTFSRCV	TVHGSNGLLI	600
KDVVGYNLSG	HCFFTEDGPE	ERNTFDHCLG	LLVKSGTLLP	SDRDSKMCKM	ITEDSYPGYI	660
PKPRQDCNAV	STFWMANPNN	NLINCAAAGS	EETGFWFIFH	HVPTGPSVGM	YSPGYSEHIP	720
LGKFYNNRAH	SNYRAGMIID	NGVKTTEASA	KDKRPFLSII	SARYSPHQDA	DPLKPREPAI	780
IRHFIAYKNQ	DHGAWLRGGD	VWLDSCFRG	EAQEGFLLTG	MKAGGILLGG	DEAASGMAQG	840
FSPPCRCLK	LVTTGSPFAH	VSLAHS				